

An abstract, vibrant image featuring a complex network of glowing, translucent structures in shades of red, pink, and blue, resembling biological tissue or a vascular system. The structures are interconnected, with some showing a grid-like pattern, suggesting a scaffold or lattice. The background is dark, making the glowing elements stand out.

TISSUE ENGINEERING

THIRD EDITION

Editors

Jan de Boer

Clemens A. van Blitterswijk

Assistant Editors

Jorge Alfredo Uquillas

Nusrat Malik



Tissue Engineering

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ACADEMIC PRESS

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Academic Press is an imprint of Elsevier
125 London Wall, London EC2Y 5AS, United Kingdom
525 B Street, Suite 1650, San Diego, CA 92101, United States
50 Hampshire Street, 5th Floor, Cambridge, MA 02139, United States
The Boulevard, Langford Lane, Kidlington, Oxford OX5 1GB, United Kingdom

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ISBN: 978-0-12-824459-3

For information on all Academic Press publications visit our website at
<https://www.elsevier.com/books-and-journals>

Publisher: Stacy Masucci
Acquisitions Editor: Elizabeth A. Brown
Editorial Project Manager: Pat Gonzalez
Production Project Manager: Kiruthika Govindaraju
Cover Designer: Miles Hitchen

Typeset by TNQ Technologies



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Preface

I was trained as a molecular cell biologist and did my first experiments on unraveling molecular mechanisms such as TGF β signaling and nucleotide excision repair. Technology seemed something not closely related to my field; after all, wasn't it all about biology? Now, more than two decades later, I realize that nothing could be further from the truth. After all, where would molecular cell biology be without the fluorescence microscope, without next-generation sequencing or machine learning? Most, if not all, molecular cell biological discoveries are driven by technological innovation.

I've been working in the Tissue Engineering field for two decades now, and I'm still in love with this field because it's exciting. Technology is not only driving the discoveries in this field, technology is part of the solutions we create. Think of bioreactor systems for the expansion of stem cells, nanoparticles that are used for the controlled release of medicines or bioactive surfaces for improving the immune response of the body. Technology is the solution to biomedical challenges.

It requires an even greater interweaving of research effort by engineers, biologists, physicians, and data scientists (to name a few). It is in this spirit that we have compiled this book, which we hope will be used to train a new generation of researchers.

As a cell biologist I got my foundation from Alberts' *Molecular Biology of the Cell*; engineers were raised with the *Physics* book by Serway, the *Engineering Mathematics* book by Kreysig, and chemists with the *Organic Chemistry* book by Wade.

Inspired by these iconic books that have made a huge contribution to the education of thousands, we decided in 2003 to put together a textbook *Tissue Engineering*. In the process of compiling the third edition, the assistant editors and I have held weekly meetings to brainstorm about how to make this book even more useful as an educational tool, and we went through the contributions of the great team of authors who have actually written the chapters with our new concepts and ideas in mind. We are extremely grateful to them for all their efforts. Now in 2022, we are happy and proud that the third version

reads as an education book, with many teaching and learning resources for students and teachers. The third edition of *Tissue Engineering* will seem familiar to those who know the second one. The order in which the chapters are presented has not changed much. We start with chapters that cover the basic science relevant to tissue engineering and then we present those chapters with applications to specific topics. However, besides some change of authorship and updated content, we have added new elements that will help “both” student and teacher to use the book as an educational tool to teach the Tissue Engineering concepts and principles. We, the editors and authors, have used the previous editions of this book in our teaching duties, and based on that experience, we added interesting and useful educational aides. First of all, now each chapter has a glossary for students to expand and learn the most relevant terms and concepts. Teacher can use glossaries to skim over potential topics of education. Furthermore, with each chapter we provide access to a downloadable PowerPoint presentation containing all the figures and captions. Teachers can use these presentations to create their lectures, and students can use them in study sessions. At the end of each chapter, we have included an extensive list of questions, for examination or debate, as well as a Challenge Based Learning (CBL) module to apply the chapter’s content into solving relevant biomedical problems. Together with the CBL instructors’ guide, these CBL modules can be used to train students in the interdisciplinary and applied skills required to further the field of tissue engineering. Thus, we sincerely hope that the book proves to be a valuable tool for the training of the new generation of tissue engineers.

Finally, this book is dedicated to the thousands of scientists and engineers who have worked in the field of tissue engineering over the past decades and whose work forms the basis of this book.

Jan de Boer

Eindhoven, October 14, 2022

An introduction to tissue engineering; the topic and the book

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1.1 Learning objectives

After reading this chapter, you will be able to:

- Describe the authors' and editors' motivation to compile this book as an educational tool to teach tissue engineering as a university-level course.
- Identify the origins of the tissue engineering field as well as its limitations and potential.
- Explain the attitude and habits tissue engineers in-training need to adopt to successfully learn and apply tissue engineering concepts and principles.
- Understand the components of each chapter to maximize learning and applying its content.

You need to understand something before you can create something.

C. van Blitterswijk, 2021.

Definitions are at best a guideline, tissue engineering is not an ideology.

One of the MERLN Institute founders during a brainstorm session in the editor's back yard, 2021.

1.2 What inspired you to pick up this book?

Welcome to the third edition of the book *Tissue Engineering*! We hope that as you read this and other chapters, you will learn a lot about this young yet dynamic field. By reading it, you will gain an understanding of the fascinating interdisciplinary approach that tissue engineers use to achieve their goal of manufacturing tissues to help patients. We hope that the content of this book inspires you to use your newly acquired knowledge in your own research.

You may be reading this chapter because you are a student enrolled in a Tissue Engineering (TE) or Regenerative Medicine course. Maybe you are a Ph.D. student looking to read up on this field for future research. Or perhaps you are a physician who wants to gain insight into the possibilities that tissue engineering can offer to better treat and diagnose your patients. Regardless, it may be true that you have come across the term *tissue engineering* before. You may have seen the video of “the mouse with a human ear”: the classic experiment where Vacanti, Langer, and coworkers implanted a biocompatible material under the skin of the back of a mouse. You may have also seen a short video of beating heart

muscle cells made from embryonic stem cells or induced pluripotent stem cells (iPSCs). It is fascinating to realize that it is possible to allow a few simple skin cells from your oral cavity to mature into stem cells and then grow them into mini-hearts. You may have also seen organoids: small pieces of gut, for example, that morph spontaneously from gut stem cells.¹ Or maybe you have heard about osteo-inductive ceramics^{2,3} which are microporous materials made of calcium and phosphate ions capable of maturing stem cells into bone cells and are being used in thousands of patients to treat bone defects. All these are examples of tissue engineered strategies being used to understand and treat diseases.

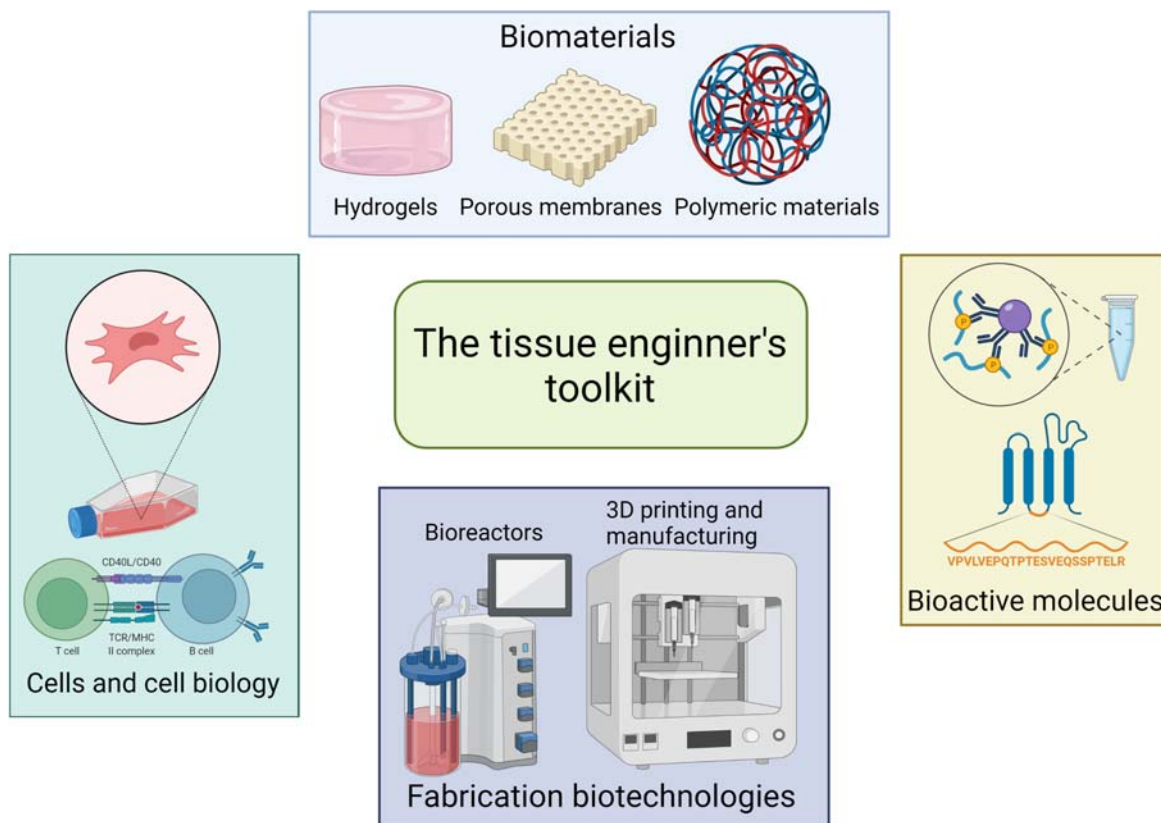
What is our reason behind compiling the *third edition* of this book? The authors who contributed to this book, whose names can be found at the top of each chapter, have been working in this field for a long time. Most of them, as did we, started their scientific education in an adjacent area (e.g., chemical engineering, mechanical engineering, electrical/computer engineering, cell biology, human biology, or medicine) but switched to tissue engineering research at some point. By working in this field, we have come to realize that the field's progress is dependent on how much we look beyond the boundaries of our own disciplines. In other words, tissue engineering is an interdisciplinary field. Just as traveling teaches you about other countries, other cultures, and other people, working in tissue engineering can also be quite educational. And just like traveling, working in other fields is also addictive. The first glance an electrical engineer takes at a fluorescently labeled cell does wonders to his/her mind and creativity. And how cool is it when you see your colleague design a knee on a computer and then printing it and then print it out to be used as a prosthesis? We tissue engineers like to look into the kitchen of others and are not afraid to roll up our sleeves and lend a hand. We, the authors of the chapters in this book, want to share our enthusiasm for tissue engineering with you and help you on your way to become a part of it.

1.3 What is tissue engineering about?

As a student approaching this course, you may have questioned: what does “Tissue Engineering” mean? You will certainly find different variations of the same definition, but one from Robert Langer, a founding scientist in the field, seems to be very accurate⁴:

[tissue engineering is] “an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ.”

Knowing the name of the game is one thing, knowing the players and what drives them is equally important. Engineers are problem-solvers by nature. Thus, tissue engineering (TE) cannot exist without the driving force and the need of solving a clinical problem. Tissue engineers will strive to find improved, new, and efficient therapies and treatments so clinicians have better tools to solve a clinical problem. Tissue engineers must be able to define the clinical problem, borrow concepts from biology, engineering and medicine, and design experiments to develop a novel medical intervention. Therefore, tissue engineers should use and develop effective tools that shall be at the clinician's disposal to improve the patient's life. Examples that illustrate tissue engineering state of the art to aid clinicians in treating patients are strategies to recover injured human lungs using extracorporeal cross-circulation,⁵ electrical neurostimulation of the human spinal cord to restore trunk and leg motor functions after complete paralysis,⁶ clinically validated one-step cartilage repair strategies,⁷ and the first pig-to-human heart transplant.⁸

**FIGURE 1.1**

General scheme of the tissue engineer's toolkit. Created with [Biorender.com](https://biorender.com).

At this point you might ask, what are the tools tissue engineers have to work with? The tissue engineer toolkit typically comprises the following: (i) biomaterials, (ii) cells (or in some cases ex vivo tissues and organs) and cell biology, (iii) biologically active molecules, and (iv) manufacturing and fabrication (bio)technologies (Fig. 1.1). You will learn about the nuts and bolts of these tools throughout the chapters of this book, as well as how to use them, whether alone or in combination, to study and hopefully solve a pressing clinical problem.

1.4 Tissue engineering's origin and progression over time

Tissue engineering approaches have their origin in the mid-70s when Langer and Folkman used polymers for the sustained release of drugs to prevent tumor growth.⁹ This work was followed by studies in the 80s when different labs around the world placed cells on substrates so they could study cell-material interactions and tissue growth.^{10–14} These observations brought excitement and interest from different research fields who rarely collaborated among each other, into what became the foundation of one of the most interdisciplinary fields of modern science: tissue engineering.

After more than 3 decades fueled by research and development, the discovery of new technologies and methods, all aiming to regenerate tissues and organs, it seems fair to ask ourselves what is really *tissue engineering* beyond its original definition. It is clear that when working in tissue engineering, we create something tangible and we make a product that is to be implanted back in the human body. In combining biological and engineering principles, we have to combine the fundamental understanding of how biological signaling pathways are regulated, how cells are differentially activated in tissue morphogenesis, and how engineering design principles are used to support those biological principles. In doing so, restoring the original tissue function with a new biological construct/substitute that can be enduring in time and integrate with the surrounding tissue becomes the ultimate goal.

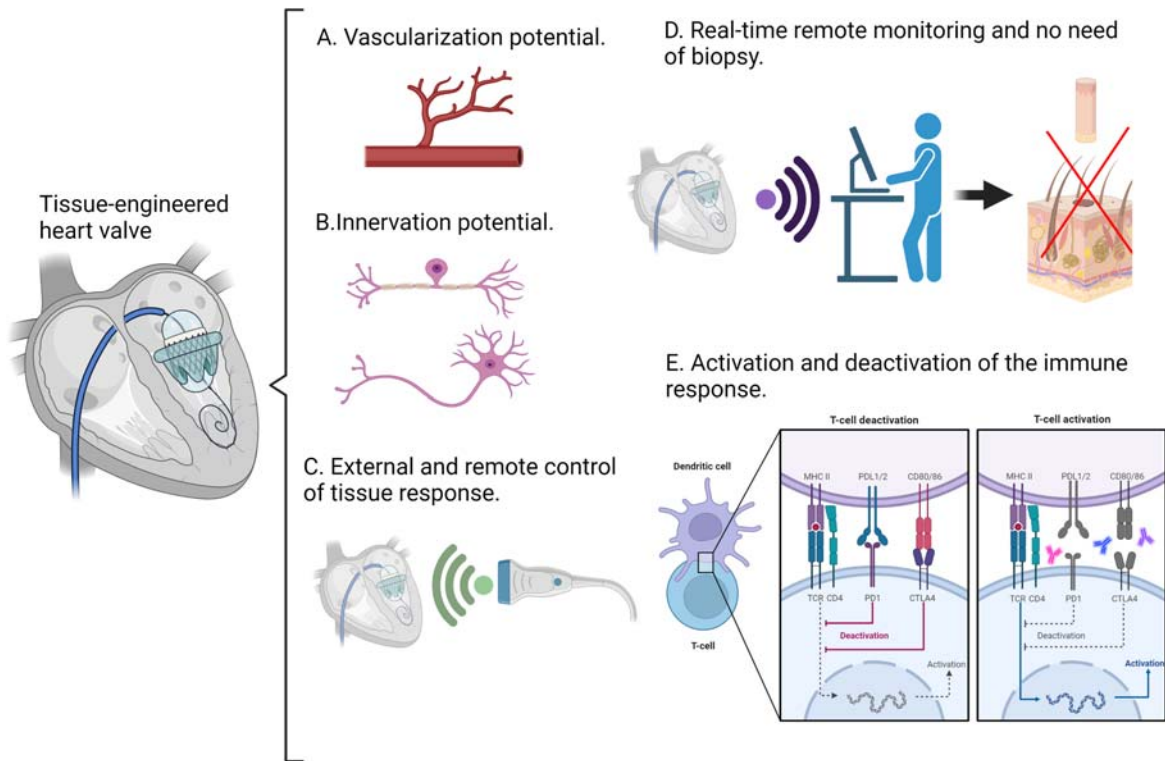
From its early days, tissue engineering has naturally evolved thanks to the breadth and depth of knowledge we have gathered over the years. Techniques to control and automate cell culture, which brought cell therapy products to the clinic such as autologous chondrocyte implantation, to the more fundamental understanding of biological principles occurring at the cell-material interface, have enabled us to design and engineer biomaterials in a more instructive manner. Nonetheless, not only the understanding of biological principles has been fundamental in tissue engineering. Tissue engineering has evolved, and hundreds of techniques and systems have been introduced to the field, making it interdisciplinary, yet complex. Nowadays, we have techniques to genetically modify cells and their transcriptome, to automatically culture and grow cells, and to bioprint with bioinks functional tissue and organs with their corresponding vasculature and innervation.

Every chapter in this book is written with a bottom-to-top approach in mind. First, learning objectives and basic principles, concepts, and definitions are explained, then examples are discussed to show how researchers use these principles to solve a specific (bio)medical problem. Finally, a challenge is presented to give you an opportunity to wear your *thinking hat* and propose novel solutions to chapter-related medical questions. Each chapter is unique; we want you to pick up where the state of the art was left off so you can be responsible of moving the field forward.

1.5 Tissue engineering's limitations and promises

About 25 years ago, the scientific world was surprised by the Vacanti mouse.¹⁵ Here, a synthetic and biodegradable polymer was seeded with bovine articular cartilage chondrocytes and implanted in immunologically incompetent mice. This proof-of-concept work demonstrated that one could take a biomaterial of choice, a cell of choice, and an animal model of choice and prove that as long as there was new tissue formation in situ, the strategy had the *potential* to be used in the clinic. Many tissue engineers and material scientists turned their efforts to discover and find applications to natural and synthetic materials, cells, fabrication processes, and biomolecules to use them, in combination or alone, to mitigate or solve hundreds, if not thousands of clinical problems. This concerted effort expanded exponentially the tissue engineer's toolbox and opened a new and exciting study field to many of us.

This book aims to update the discussion on concepts, principles, and definitions relevant to notorious advances in the field over the past 10 years, while explaining about major shortcomings that need to be solved before we can move the field forward. As you will read in the coming chapters, there are still many drawbacks and limitations to resolve before we can translate many laboratory-ready therapeutics

**FIGURE 1.2**

The most pressing and contemporary problems with tissue-engineered organs and tissues. An engineered heart valve is used as example. Created with [Biorender.com](https://biorender.com).

to the clinic (Fig. 1.2). One of the most pressing problems in the field, which is still a work in progress, is to provide engineered tissues and organs with functional vascularization and innervation. Additionally, we often engineer tissues without paying enough attention to the disease environment. Our body's immune response should take central role in future tissue engineering approaches, not only to minimize or control the foreign body response, but to better understand the acute or chronic inflammation that can be triggered when tissue-engineered therapeutics are implanted. Furthermore, when an engineered tissue is implanted, we do not know how it evolves until a biopsy is taken. Methods to keep monitoring the evolution of an engineered tissue should be further developed and combined with engineering principles that could result in adjusting the tissue in real-time if needed (e.g., via the application of external and remotely controlled stimuli). These and many other needs and design limitations were not envisioned when the field was in its nascent stages. It is up to you, the tissue engineer in-training, to find viable solutions.

With knowledge comes great responsibility. Looking back at the Vacanti's mouse model, for example, one would now argue that the synthetic polymer is not placed in an anatomically relevant location, or that an athymic mouse does not provide important information about the interaction between the

immune system and the implanted material. These questions would be followed by inquiries about how well vascularized and innervated the implant is. Queries on scalability of the manufacturing process, or ethical and regulatory considerations that one would have to implement early in the manufacturing process, are questions that were not on the tissue engineer's radar 25 years ago. Nowadays, they are and have to be answered in an interdisciplinary manner. Biologists, bioengineers, and medical professionals should actively exchange ideas, strategies, and techniques to comprehensively answer complex tissue engineering questions. We hope that you, after reading this book, can understand and apply its content, so you can thoroughly assess the real clinical potential of tissue-engineered therapies.

The initial success and excitement in tissue engineering was a strong driving force to move the field forward. Nonetheless, there are still many technical and scientific limitations that need to be circumvented. For example, using the term "tissue engineering" to perform a quick search shows that there are about 100 tissue-engineered therapies currently evaluated in the clinic (www.clinicaltrials.gov). This number is low if we compare it to the 326,000 manuscripts written on basic and applied TE science. It seems like there is a mismatch between the academic discovery of TE therapeutics and their potential to be translated to the clinic. We can find similarities with the drug discovery and development world since only 15% of all drugs who enter the human testing phase effectively reach the marketplace.¹⁶ Is the current TE pathway to bring therapeutics to market moving toward the long and expensive drug discovery model where a new drug takes approximately 12 years and \$1.8 billion to reach the patient?¹⁷ We hope not but we are not entirely sure. The regulatory landscape treats tissue-engineered therapeutics as advanced therapy medicinal products¹⁸ and imposes regulatory requisites as rigorous and stringent as those applied in drug discovery and development. Nonetheless, we expect that automation, *in silico* modeling, organoids, novel biofabrication techniques, and cell sources like induced pluripotent or mesenchymal stem cells will have a positive impact in creating a sustainable and cost-effective TE model to effectively and safely bring therapeutics to market.

TE strategies in clinical trials are gathering long-term efficiency and safety data while restoring tissue's normal anatomy and function. In the following chapters you will read, for example, that new TE skin grafts (see Chapter 15) are being engineered to have sweat glands and hair follicles to maintain normal body temperature, exude toxins, and function as a dynamic infection barrier. The new generation of skin grafts aim to regenerate diabetic foot ulcers, since these grafts are thick enough to include both epidermis and dermis and possess functional irrigation and innervation. You will also learn that there are limitations with autologous chondrocyte transplantation to fix knee cartilage defects (see Chapter 16). These techniques are not ideal because cell expansion can take several weeks, require specialized and expensive cell culture facilities, and are technically complex as autologous chondrocytes need to be combined with allogenic cells before spraying them on the defect site.

Nevertheless, in the last years, there has been important clinical success of TE therapeutics that have energized the field. Recently, researchers used iPSCs in a clinical test to treat spinal cord injuries for the first time.¹⁹ Another step forward in the field of spinal cord injury regeneration was taken not long ago when three patients with complete sensorimotor paralysis received epidural electrical stimulation (EES). EES was performed through an implanted medical device to help patients stand, walk, cycle, swim, and control trunk movements.⁶ These new successes can lead us to reflect on the immense clinical impact of tissue engineering, a field that originated only 4 decades ago.

1.6 The future of tissue engineering

This book provides a complete overview on the principles and concepts that form the bedrock on which tissue engineering lays. This book also sketches the field's state of the art with the aid of practical examples throughout each chapter. The 3rd Edition will show you how the different disciplines work together to ultimately implement tissue engineering in the clinic. Yet, what can we actually expect from tissue engineering when it comes to the future, by the time you will be contributing to this field? Which projects extracted from your own research will appear in the 7th or 10th edition of *Tissue Engineering*? Making predictions is very difficult, and also a subject this field has struggled with. We will try to do our best to predict major future milestones hereafter in this section.

In the early days of tissue engineering, envisioning progress was easy, and we were very optimistic. Progress relied on isolating some cells from the body, fabricate a scaffold, and implant it in the patient. How hard was that? Not too much. That early naivety has now been replaced by realism for the seasoned tissue engineer. For those outside the field it has also been a reason to be skeptical, and some dismissed TE as a hype. We think it may have been one of the reasons why the term *tissue engineering* went out of fashion at some point, and why people started using the term *regenerative medicine* as an alternative. We think that is a shame because the term tissue engineering covers our work well and has demonstrated radical and meaningful success over the years. What can we realistically expect from the tissue engineering field in the years to come? It is safe to assume that allogeneic islets of Langerhans will be used in type-I diabetic patients, just as heart valves grown in the body through in situ tissue engineering will become a clinical reality. In these cases, researchers are already very far in the preclinical development, and it seems only a matter of time to embark on the cautious path of clinical development. Xenotransplantation, or transplantation of animal-derived organs in humans, is another area where the first clinical success of a genetically modified pig heart implanted in a human recipient was achieved.⁸ Attention and efforts should be placed in the ethical, scaling-up, and manufacturing considerations to broader the impact of this technology in the clinic.

We deem it less realistic that large organs will be produced by means of tissue engineering. On the one hand, it is realistic to assume that the cultivation of cardiomyocyte sheets from iPSCs will be successful and that these tissue sheets will be used to repair a local heart defect. On the other, it is not realistic to assume that we will be able to grow or bioprint a fully functional heart from cultured cells and bioinks. The reason for this is that the heart is an organ that matures in the embryo's body over a period of weeks to months and in which maturation is dependent on the proper incorporation of blood vessels and nerves. With the current knowledge and state of technology, we do not see how this can be achieved. The same also applies to other complex organs such as the kidney, the liver, and the brain. Local adjustments and fixes to the existing damaged organ are now possible, but constructing whole organs is beyond the horizon. This claim is firm, but also grounded in realism stemming from decades of work on musculoskeletal tissue engineering research. Our first attempts at tissue engineering were in the field of bone replacement: there is preclinical evidence that cell-based tissue engineering works, and bone replacement based on biomaterials or growth factors has been widely used clinically. Yet, this always involves relatively small pieces of bone, up to 1 or 2 cm at the most. Large bone defects, which must also be capable to withstand loads, cannot be treated with tissue engineering even after extensive work in this field for 30 years. This also leaves 3D printing of humans, as presented in the

movie *The Sixth Sense*, in the realms of science fiction. Still, science keeps on surprising us. New techniques are being invented that could bring about a paradigm shift and undermine our proposition. In fact, we hope they do.

1.7 Tissue engineering and you

The fact that you have picked up this book and read this far shows that you are ready to take steps into your training as a tissue engineer. How do you make the transition from a recent high school graduate to someone who is learning at the bachelor's level? How will you transfer the experience and knowledge you are acquiring when it comes to start working in this field? What kind of work is out there anyway? With regard to the latter, a look at their *LinkedIn* profiles shows us that our undergraduate and graduate students have ended up in a wide range of jobs. Among our former students are surgeons, medical device experts, a CEO of a biotech company, clinical physicists, policy makers, and university professors. Each of them takes the knowledge from her/his education to apply it in a particular field, from policy to product development, and from project management to education. After completing their education, each of them had to learn new things that biomedical engineering does not include in its curriculum. Lifelong learning is the adage, and this is the mindset tissue engineering will require from you from now on. As mentioned above, tissue engineering is an interdisciplinary field and everyone in it struggles to keep up with new developments and techniques. We certainly do. In one tissue engineering area you can be an expert, while in another you are a true novice. We experience this duality every day because we are in conversations with fellow scientists and students from other disciplines who want to work with our research groups, who want to follow our education, or who want to collaborate with us.

With this in mind, we can advise you that the tissue engineer in-training should be able to master not only the understanding of biological principles but their combination with engineering and fabrication principles to model diseases and organ systems. In using this book, you will see that a thorough and profound understanding of a tissue engineering challenge requires you to have background knowledge in basic medical sciences (anatomy, physiology, pathology, histology, and medical imaging), basic biological sciences (cell and molecular biology, and embryology), and natural sciences (basic engineering principles, mathematics, physics, and chemistry).

As a tissue engineer you will have to adopt and develop a dual mindset: that of the scientist and that of the engineer. As a scientist, developments are hypothesis-driven, and you will have to design experiments and execute them rigorously. As an engineer you will have to make quick “back-of-the-envelope” calculations and perform pilot experiments to try new ideas. You will need to try fast and fail faster. Working in tissue engineering will require you to learn to work and mentally switch between scientific and engineering methodologies. As you may know, experimentation and science can, and will, have many unknowns. Most of your time will be shared between failed experiments and data that is unclear and needs further processing. As a tissue engineer, you need an analytical and critical mindset. Why do I need to perform this experiment? What information will I get from it? How important are the experimental results to reach to meaningful conclusions? Are these experimental conclusions positive or negative to move forward with the project? Most importantly, as a tissue engineer, you will learn to work in a team. You will learn to ask the right questions to experts and be smart enough to integrate their advice into your project. You will delineate your path and grow as you

move forward within the field. You will learn to cross boundaries to other fields and borrow knowledge from them. The path is long but exciting and full of interesting treasures waiting to be found.

1.8 How to use this book? A guide for students and teachers

This book was written with 2nd and 3rd year bachelor students in biomedical engineering or biological sciences in mind, because these were the students we were teaching during the brainstorming session for the first edition of this book in 2003. They are also the students we still offer education today, although we also teach students in the regenerative medicine bachelor and master's course, and medical engineering and technical medicine bachelor courses.

We hope that this book will be used in the curricula of medical students who wish to enroll in this field in preparation for a specialization and also by graduate students who come from another field to work and study tissue engineering. When we initially compiled the chapters of this 3rd edition, we also considered that different users with different levels and different objectives will use this book. Therefore, each chapter starts with the basics and goes more in depth as you continue reading it.

Each chapter contains basic knowledge that is needed to introduce the main topic. At the beginning, the content does not go very deep, and the knowledge is explained in the context of tissue engineering. Each chapter has examples of what we consider to be important and insightful developments on the topic. The chapters are explicitly not intended to be a comprehensive review of the field, but rather an explanation of concepts and principles on which the field is positioned on. In the text, we refer to articles that are deep and broad enough to serve as educational tools. Each subject and field uses its own jargon, and certain concepts overlap between chapters, such as, for example, the cross-talk in signal transduction to understand immunology tissue engineering and its impact in modulating the foreign body response. At the end of each chapter, the authors have compiled concepts and jargon (in the form of a glossary) to provide the reader with an easy-to-use guide to learn the chapter's content. The glossaries and the end-of-chapter questions can be used also by the teacher to design tests.

The topics in this book were carefully chosen and laid out in a logical sequence to draw the path from basic knowledge to clinical implementation. Coeditor Clemens van Blitterswijk expressed the need for basic knowledge in the statement: "you need to understand something before you can create something." Creating is certainly a basic activity of the tissue engineer, which is why the book starts highlighting the basics of biology in the fields of cell signaling, embryology, stem cells, and the extracellular matrix. These concepts are followed by basic material science concepts and principles in synthetic biomaterials, degradation of biomaterials, and the important cross-talk between the two fields when it comes to understand cell-material interactions. This book then focuses on the technology needed to produce and fabricate the building materials: biomaterials discovery, microfabrication, scaffold design and fabrication, and controlled release. The next chapters can be seen as *true* tissue engineering chapters. They deal with the clinical challenges and the techniques that can be used to meet and solve those challenges. The last two chapters take a closer look at controlling the process from idea to clinical implementation.

As a summary, each chapter is an anthology of the topic being discussed but it does not represent, by any means, a complete compilation of facts and discoveries. The suggested readings at the end of each

chapter will help you to take some extra steps to understand the topic in more detail but after that, you and your search engine are on your own to explore. The book has 21 chapters, and the choice is up to the reader or the teacher to cherry pick: reading that one chapter that you just want to know a little more about or read and understand a large number of chapters as part of a course curriculum.

1.9 How to use the chapters?

Each chapter has a similar structure, which facilitates reading and teaching the content (Fig. 1.3). It starts with an abstract, and one or two classic quotes from leaders in the field. Then a few learning objectives are laid out to highlight what the student should master to become proficient in the topic. The learning objectives are a cheat sheet for the teacher and student to see if they have already achieved their goal or to help define teaching modules. Within the main text, important words are **bolded** to show the reader the concepts and principles he/she needs to focus his/her attention on. All these bolded terms are compiled at the end of the chapter in the glossary. The glossary is a tool for learning the language of the subject and as a handle for formulating questions. If the student needs to dive in deeper into the topic, then we recommend becoming familiar with the recommended readings and the list of references. The questions at the end of the chapter can be used by the students to see if they have understood the content and as a means for the teacher to initiate discussions in class. To apply the knowledge the student has gathered while reading the chapter, we have generated a challenge-based learning (CBL) module that is presented as an opportunity to creatively generate new solutions to pressing biomedical problems. In short, CBL is a problem-based learning method in which students work in groups to solve a specific tissue engineering case. On the basis of predefined questions, the students read up on the subject and discuss their acquired knowledge among themselves and under the supervision of a tutor. Then the students come up with a

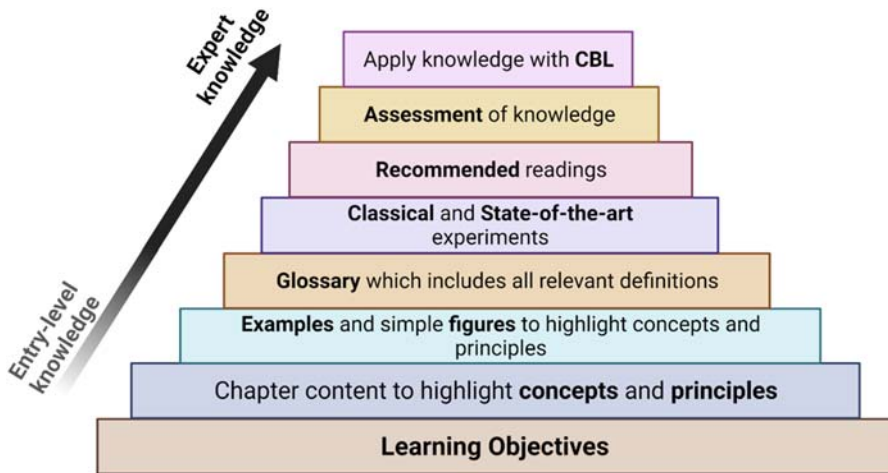


FIGURE 1.3

The structure of each chapter is designed to understand, analyze, evaluate, apply, and ultimately create new Tissue Engineering knowledge. Created with [Biorender.com](https://www.biorender.com).

solution for the problem, which they further develop and present. CBL is a proactive and open-ended way of learning that in our experience encourages students to find, absorb, and understand new knowledge. We wish you a lot of fun reading and using this book. Who knows, maybe thanks to this book we will become colleagues and we will meet at a tissue engineering conference, work as collaborators in a project, or serve as committee members in a grant review session. Enjoy!

Summary

- Tissue engineering's progress is dependent on how much researchers look beyond the boundaries of our own disciplines, as the field is inherently interdisciplinary.
- Every tissue engineering challenge aims to solve a relevant clinical problem, and tissue engineers have biomaterials, cells, biologically active molecules, and (bio)fabrication methods to tackle the problem.
- One of the main challenges in tissue engineering today is to provide tissues and organs with functional irrigation, innervation, and the capacity to modulate the immune system.
- Tissue engineers in-training need to behave as a competent scientist and a resourceful engineer.
- Each chapter has many educational tools that will be equally useful for students and teachers to acquire novel tissue engineering knowledge.

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Stem cells

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2.1 Learning objectives

After reading this you will be able to:

- Learn the defining properties of stem cells.
- Appreciate the broad scope of available stem cells, their diverse gene expression that makes them useful, and their differentiation potentials.
- Become familiar with the methods, assays, and skills needed to study stem cells.
- Appreciate the stepwise phenotypic analyses necessary to characterize successful stem cell differentiation toward a desired tissue.
- Envision the challenges and opportunities for tissue engineers using stem cells.

It is a riddle, wrapped in a mystery, inside an enigma: but perhaps there is a key.

W. Churchill, 1939.

We know more about stem cells today than at any previous time, yet the pace of new discoveries continues to accelerate.

M. Pittenger, 2022.

2.2 Introduction

Early development starting from the fertilized egg (zygote) is a time of intense cell proliferation. The zygote then forms the morula, a solid ball of cells which then forms a fluid-filled space termed the **blastula**, where the outer layer is composed of trophoblasts that will form the placenta and an inner cell mass that will form the embryo. The **inner cell mass** of the developing embryo forms on one side of the blastula, and the cells at this stage have about equal contribution to the developing embryo—they are all **stem cells**. Once the embryo has 120–200 cells, the embryo changes form and the cells start to differentiate. The outer layer of cells will become the **ectoderm** and there is an invagination that marks the beginning stage of gastrulation from which the presumptive gut and its related tissues will form—the **endoderm**. The cells between the inner and outer cell layers are known as the **mesoderm** and will form most of the internal organs. However, at this early stage, cells from one presumptive layer are not yet determined to be ectoderm, mesoderm, or endoderm as transplanting cells from one layer to

another, the cells will adopt the phenotype of the layer to which they are transferred. Nevertheless, these three layers of early cells can be considered the stem cells from which all tissues are produced. Some of these stem cells appear to be retained after birth and may last into adulthood, but they become rare in most tissues.

Stem cells can proliferate and differentiate to become cells with new phenotypes and functions. As such, they provide a useful cell type for many tissue engineering applications. They provide certain challenges as well. The field can be divided into adult, embryonic and induced stem cells, but may also be further subdivided in additional ways. For the purposes of this book, we are interested in those stem cells that can be isolated, manipulated, analyzed, combined with other materials, further engineered, and placed back into the *in vivo* environment to perform a specific function. We are still discovering many aspects of stem cells, but stem cells and tissue engineered constructs containing them are currently undergoing preclinical and clinical evaluation in many areas of medical science.

The cells, tissues, and organs of the body are under constant use and many cells die and are replaced by new cells on a daily, weekly, or monthly basis. **Homeostasis** requires the loss of cells to be accurately balanced by cell replacement. Injury and repair/regeneration can accelerate cell replacement, but aging causes this process to slow. Understanding of normal cell replacement and its relationship to injury repair is a goal of modern medicine and the tissue engineer. Every day, millions of blood cells die, and millions are created to replace them. Individual white blood cells live for 1–3 days, and all your red blood cells are replaced every 120 days by **hematopoietic stem cells (HSCs)** in the bone marrow. Skin cells are replaced frequently from epithelial stem cells in the basement layers and migrate to the surface as the outer differentiated cornified epithelial layer slough off (see Chapter 15 Skin engineering and keratinocyte stem cell therapy). Your whole skeleton is replaced every 6–8 years by the combined actions of the osteoclasts which remove and remodel the calcified bone matrix, and the osteoblasts which divide and then differentiate into new osteocytes and produce extracellular matrix which becomes cross-linked and calcified over time (see Chapter 16 Cartilage and bone regeneration). The central nervous system—brain and spinal cord—was thought not to regenerate but research over the last 15 years has shown the cells of these tissues may renew slowly (see Chapter 17 Tissue engineering of the nervous system). The heart was thought to contain the same cells from birth to death and only recently has evidence emerged for new cardiomyocytes over time (see Chapter 18 Principles of cardiovascular tissue engineering). However, cardiomyocyte turnover is very slow, and a 50-year-old person has replaced only about half of his/her cardiomyocytes present after their first birthday. These examples demonstrate the potential of stem cells to produce new healthy cells as needed by the body but there are limitations. In this chapter, we will examine further the properties of stem cells and some of their characteristics that make them useful for tissue engineering. Understanding the signals which promote proliferation of stem cells and their subsequent differentiation to functional cell types has become a rapidly advancing field that stretches across many disciplines—cell biology, biochemistry, molecular biology, bioengineering, material science, and many medical specialties of cell (hematology/oncology), tissue (pulmonology, cardiology, neurology, orthopedics, dermatology, etc.), and organ systems (internal medicine, gerontology, etc.). The study of stem cells is not new, but recent advances have brought great interest and the promise of using stem cells for tissue repair and spawned the rapidly advancing field of regenerative medicine. **Regenerative medicine** strives to utilize and enhance the body's natural regenerative potential to repair and return damaged tissue to its full functional capacity.

In Fig. 2.1, the abundance and range of human stem cells is depicted. The sperm and egg form a zygote that grows to form the blastocyst with its inner cell mass from which **embryonic stem cells (ESCs)** can be isolated. Alternatively, adult somatic cells can be isolated and reprogrammed by transfection with a combination of immortalizing genes to produce the **induced pluripotent stem cells (iPSCs)**. The ESC or the iPSC can produce cells that form ectoderm, endoderm, and mesoderm, and these major lineages can foster the production of more differentiated cells with more specific functional attributes. As the cells become more differentiated, they lose potential for other lineages but gain functional attributes as depicted to the right and left sides of the figure. The arrows between cell lineages in this figure should be thought of as changes in gene expression that delineate the steps in cell lineage **differentiation**. The efficiency of each step is not high in vitro, and often limited numbers of differentiated cells are produced.

2.3 What defines a stem cell? Self-renewal, proliferation, and differentiation

Stem cells are not easy to identify. They look like many other cells, and they may be present among cells that are not stem cells. The two daughter cells from the division of a stem cell may behave as stem cells or one or both may have already begun the process of differentiation. The “choice” is not always

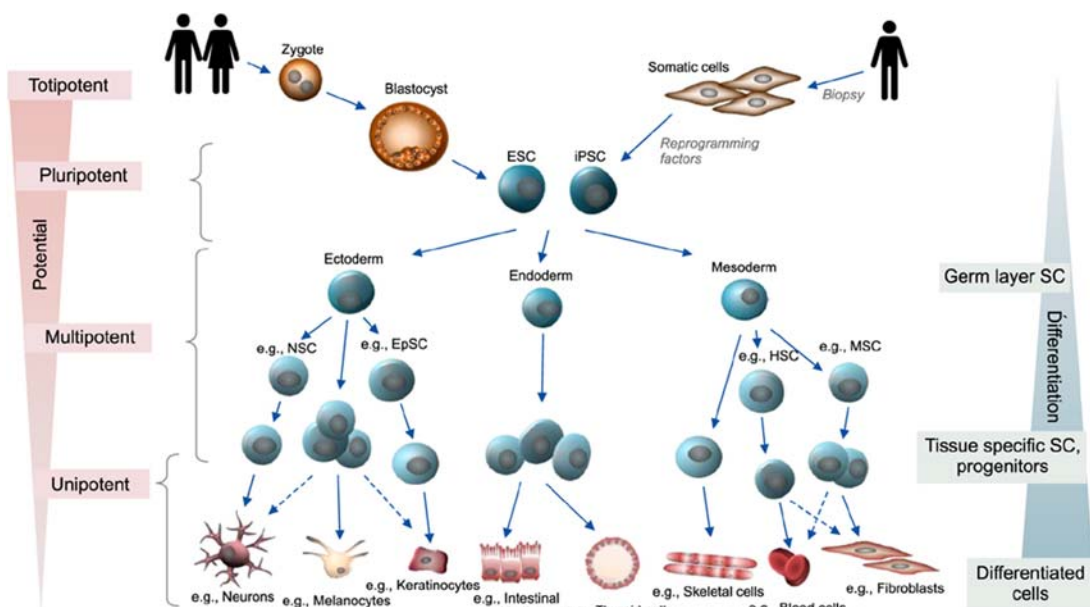


FIGURE 2.1

Classical hierarchal model of stem cell differentiation. As pluri-potential goes down (left side), the cells undergo a greater degree of differentiation (right side). *ESC*, embryonic stem cell; *EpSC*, epidermal stem cell; *iPSC*, induced pluripotent stem cell; *HSC*, hematopoietic stem cell; *MSC*, mesenchymal stem cell; *NSC*, neural stem cell. From Prodinge CM, Reichelt J, Bauer JW, Laimer M. Ann Dermatol. 2017;29: 667–687.

evident. In general, stem cells in the body may be slow to divide and most are thought to be quiescent, preserving their potential until needed. Once a stem cell begins to divide it may produce daughter cells that are given names, such as **progenitor cells (PCs)**, **transit amplifying cells (TACs)**, early differentiated cells, and fully differentiated cells, which reflect the loss of proliferative potential and the gain of functional attributes.

2.4 Self-renewal

Self-renewal is a special form of cell proliferation. **Self-renewal** refers to the ability of a stem cell to divide and make identical copies of itself to assure that the stem cell population is not depleted, and exists throughout development, and some may remain for the life of the organism. Self-renewal in vivo requires a specialized location—the **stem cell niche**—where stem cells are maintained through the action of support cells nearby. In vitro conditions that approximate conditions found in the stem cell niche have been developed, but these conditions are usually imperfect and may not be as favorable as those found in vivo. This remains an area of active study. Self-renewal allows proliferation of stem cells, or it can produce one daughter cell that is a stem cell and one daughter cell that then proceeds along a differentiation pathway. This asymmetric division may be caused by cell fate transcription factors accumulating unequally in one daughter cell or can be caused by signals outside the cell such as another cell or a gradient of morphogens, or growth factors from the nearby tissue.

In Fig. 2.2, the early proliferation of stem cells in the stem cell niche, where they are surrounded by support cells, pushes some progeny further away and leads to proliferation outside the niche where they divide, amplify, and then begin to differentiate. **Symmetrical division** of stem cells in the stem cell niche creates two identical daughter stem cells. As a stem cell leaves the stem cell niche, it may undergo **asymmetrical division**, creating two nonidentical daughter cells. The asymmetry can be caused when cell division occurs to divide the cell or its organelles unequally, or the environment provides different signals to each daughter cell. Proliferation of daughter cells as they move away from the stem cell niche can quickly amplify a population of PCs to create many cells, hence the name **transit amplifying cells**. From the transit amplifying stage, that produces largely 1 cell type, the cells begin differentiation and express genes and proteins characteristic of differentiated cells, and this differentiation and maturation is dependent on the environment. As these cells move further into new environments, new signals and cell–cell interactions can produce completely different mature phenotypes.

2.5 Stem cell proliferation

Proliferation of stem cells allows the single cell zygote—the ultimate stem cell—to grow into an adult organism. Along the way, tissues and organ systems develop and become fully functional. A subset of stem cells may self-renew and be stored in each tissue for later use in repair and regeneration. When potential stem cells are isolated from an embryo or an adult tissue, the first evaluation is usually whether the cells can proliferate and if so, how fast and for how long. The conditions for in vitro growth are found by experience and trial and error as cells become adapted to their new environment. The doubling time of stem cells in culture is usually in the 20–30 h range when conditions are favorable. Eventually, the dividing stem cells may use all the space available to them on the culture dish or

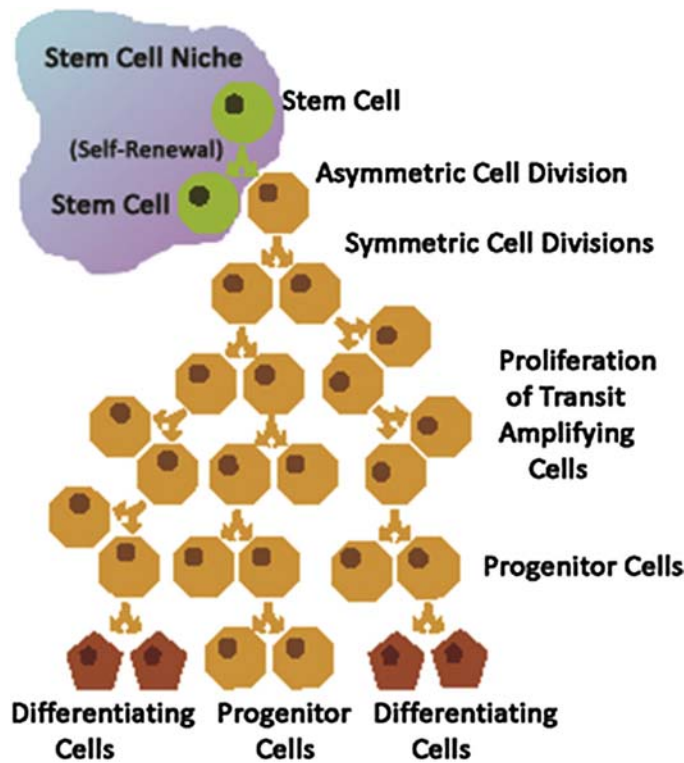
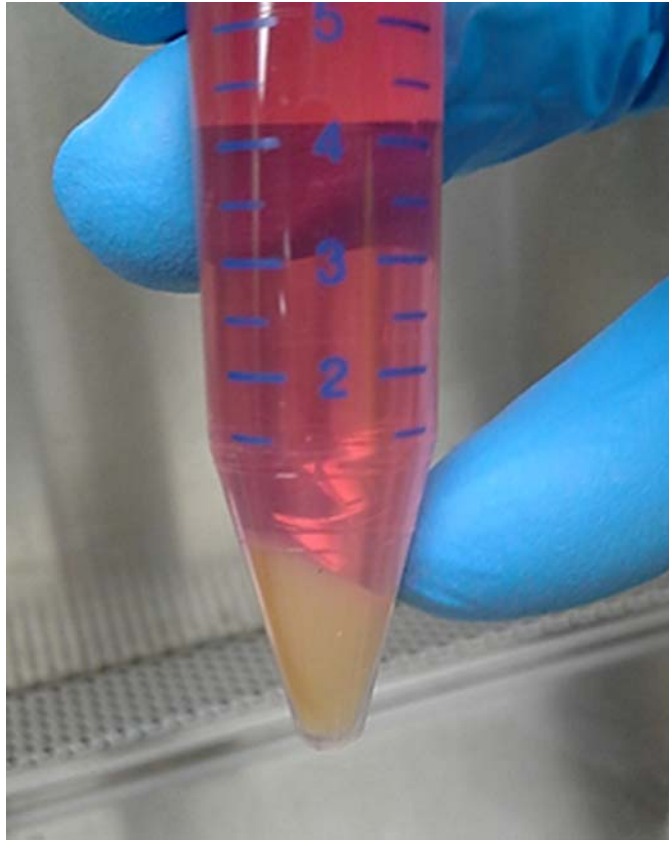


FIGURE 2.2

Stem cell replication occurs in the stem cell niche and can be symmetrical or asymmetrical, and the most rapidly dividing cells are the transit amplifying cells. A few progenitor cells may exist among the nondividing differentiated cells in the adult tissue.

metabolize all an important nutrient, and the rate of cell division will decrease to the stage of **quiescence**. At this point, the cells in the culture vessel must be subcultured into two to four new culture vessels with fresh nutrient medium. This step is referred to as “passaging” the stem cells, and each passage usually represents three to four doublings of the stem cells. Stem cells are often cultured this way to keep them in log phase growth to produce millions of cells in a predictable time period. Keep in mind that a single cell dividing every 24 h can produce 1 million cells in 21 days and 1 billion (10^9) in 31 days (Fig. 2.3). Here in this figure there are 100 million bone marrow mesenchymal stem cells (BM-MSCs) resulting from 3 weeks of culture, collected by centrifugation following mild trypsin treatment to release them from the flat surface of tissue culture flasks. A useful number to remember is that there are approximately 37 trillion cells that make up the human body. In terms of cell proliferation, one stem cell divides into two, two stem cells divide into four, four stem cells divide into eight, and so on. Therefore, 10 cell division cycles represent 1024 cells (assuming all cells survive and divide again). 20 cell division cycles represent 1,048,576 or ~ 1 million cells. 30 cell division cycles represent 1,073,741,824 or ~ 1 billion cells. As mentioned above, Fig. 2.3 shows 100 million mesenchymal stem cells after collection in about 400 μL or 250 million cells/g. From these numbers we can

**FIGURE 2.3**

Proliferation of stem cells.

calculate that a 100 kg-person has $\sim 25 \times 10^{12}$ cells. Another study by Bianconi et al. 2013 estimated 3.7×10^{13} cells (37 trillion) in the body including $\sim 3.6 \times 10^{11}$ cells in the liver, $\sim 2 \times 10^{12}$ cells in the skin, $\sim 2.5 \times 10^{12}$ cells in blood vessel endothelia, $\sim 2 \times 10^9$ cardiomyocytes, and $\sim 2 \times 10^9$ fibroblasts in the heart.

As a point of reference, the average adult human is composed of about 37 trillion cells. Ideally, stem cells can grow forever. In practice, it is difficult to keep stem cells growing indefinitely, yet enough cells can be produced for a particular purpose and the remainder may be cryopreserved for later use or discarded. Early studies on aging of cells in culture using human fibroblasts by Leonard Hayflick found that after about 50–60 divisions the cells slowed and stopped growing. Prior to this work it was assumed that cells in culture could divide an infinite number of times if the proper conditions could be found. Hayflick's work identified that normal cells were mortal and could divide a finite, limited number of times, and these normal cells would not form tumors when injected into animals. This paradigm of cellular aging accompanying cell division is still an important concept, but stem cells may have additional properties that allow them to surpass this limit.

2.6 Stem cell differentiation

The third defining property of a stem cell is its ability to differentiate into a more specialized cell. Differentiation is the process whereby stem cells become more specialized cell types and can perform new functions through the expression of new genes, mRNA, and proteins. Differentiation involves the deactivation of some genes and the activation of a new set of genes. The signals that cause stem cells to differentiate are many, and often it is difficult to keep many pluripotent stem cells from differentiating during proliferation. Differentiation can be caused by a change in basal nutrients, a change in the cell's environment, a stimulation, or a lack of stimulation of a signaling molecule, a new cell-to-cell interaction, etc. Some programs of differentiation are more common than others and some types of stem cells proceed more easily along certain lineage pathways to a "preferred" differentiated phenotype.

Stem cells are poised for change, and it can be subtle, or it can be dramatic. Over many years, a large effort has identified differentiation conditions that can be applied in vitro to the study of stem cells and their potential for differentiation. Although these conditions may work very well to initiate differentiation, the fully differentiated phenotype found in adult tissue is not often evident in vitro. That is, additional signals may be necessary for maturation of the differentiated phenotype. It is generally assumed that the signals that cause in vitro differentiation are related to those that cause the same result in vivo, but this may not always be true. Nevertheless, numerous assays have been developed that reproducibly induce stem cell differentiation to desired phenotypes, allowing further experimentation and understanding of the signals involved in the differentiation process. An important scientific parameter for understanding stem cells is their potency. **Potency** is the ability of the isolated cells to differentiate to multiple lineages—the more lineages to which a stem cell can differentiate, the more "potent" is the cell:

- **Totipotent** stem cells can form an entire organism. The fertilized oocyte and the cells after the first cleavage divisions are considered totipotent.
- **Pluripotent** stem cells can form all three germ layers including germ cells, but not the extraembryonic tissue as placenta and umbilical cord. Cells of the inner cell mass of the blastocyst are pluripotent. When these cells are brought into culture, they are called embryonic stem cells (ESCs).
- **Multipotent** means the ability to form multiple cell types. For example, mesenchymal stem cells (MSCs) can differentiate into cells that form bone, cartilage, and fat—all mesodermal tissues.
- **Oligopotent** stem cells can differentiate into two or more lineages, for example, neural stem cells that can form a subset of neurons in the brain.
- **Unipotent** is the ability to form cells of a single lineage, for example, spermatogonia stem cells.

Potency is also a parameter assigned to an agent for a particular use. Assay potency for cellular products is a quantitative in vitro assay that supports the agent's use. In the patient setting, **clinical potency** has a meaning related to the cellular products' ability to effect change in a measurable clinical parameter. Obviously, striving for assay potency and clinical potency to coincide whenever possible is desired, but this is not always directly possible. The potency of a drug is important to determine the dosage given to a patient and it must be reproducible across multiple recipients. In the case of bone marrow-derived mesenchymal stem cells (MSCs) given to a patient with graft versus host disease, the potency assay performed on the cells is a measured reduction in T cell proliferation, or the expression of one of the secreted molecules that stops T cell expansion. If the MSCs are to be used for cartilage

repair, the measured potency of the dose will be the expression of chondrocyte-specific protein expression such as aggrecan or collagen II in a standardized assay.

2.7 Stem cell quiescence and activation

In the adult, not all stem cells are needed to be constantly proliferating and many are quiescent. **Quiescence** is a stable state in which the stem cell is not in the cell cycle but retains the ability to divide at a later stage. The quiescent state is actively maintained by surrounding cells of the stem cell niche, and a perturbation of this setting can stimulate stem cell proliferation. Fig. 2.4 depicts the cell cycle with the added dimension of the quiescent state. Adult stem cells may be in a primed shallow quiescent state from which they readily enter an active cell cycle, or they may reside in a deep dormancy state that may require multiple activation signals. Once activated to divide, the stem cells may produce multiple daughter cells before exiting the cell cycle at the R-point (restriction) to become a mature differentiated cell. Quiescent stem cells in a G-zero state are found in deep dormancy or a shallow dormancy (primed quiescence) from which they may be stimulated to the G1 state and proliferate. Most adult differentiated cells are in the G1 state at the R-point and “restricted” from proliferating by external and intracellular gene regulation.

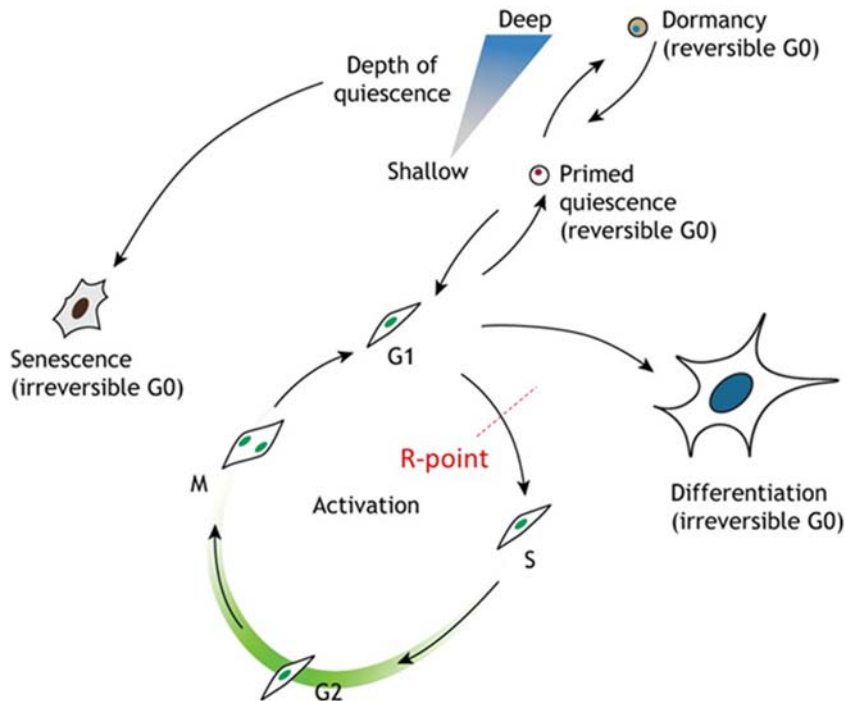


FIGURE 2.4

Stem cell quiescence and the cell cycle. From Urban, Cheung. *Stem cell quiescence: the challenging path to activation*. Development. 2021; 148:dev165084.

The idea of stimulating intrinsic tissue resident stem cells to proliferate and then cause them to differentiate is one of the current concepts for tissue regeneration. This would seem to be the ideal approach as there is limited manipulation of the endogenous cells and no issue of immune rejection. However, it has proven difficult to accomplish in situ stimulation of stem cells even in tissues where they are known to reside. With aging, many tissues are depleted of identifiable stem cells, so it is not clear if this strategy will work later in life. Still, this approach continues to see much research activity.

The quiescent state can be induced in some cells such as fibroblasts in culture where serum starvation or confluency causes the cells to stop dividing. But this nutritional or spatial quiescence is different than that of the quiescent adult stem cell which is actively maintained by factors from support cells present in the stem cell niche. There are common features of quiescent stem cells in different tissues, but there is not a common molecular marker of quiescent adult stem cells. Rather it is a metabolically low activity state of the stem cell with low levels of mRNA transcription and protein translation (proteostasis) and low cell mobility. The gene-rich heterochromatin remains tightly coiled, limiting transcription factor access to promoters. Ribosomes and mitochondria are few. In some cases, mRNAs accumulate with introns retained, ready for rapid processing when growth and differentiation signals allow. The cyclins, cyclin-dependent kinases, and their inhibitors (p21, p27, and p57 in humans) all have a role in the quiescence of adult stem cells.

A few examples of tissue-resident quiescent stem cells will aid in understanding the diversity of tissue needs of these long-lived cells. Some tissues such as the blood, skin, and intestines are actively and continually producing differentiated cells but also need to retain stem cell function late into life. Here the stem cells are renewed frequently. HSCs are continually replenished in bone marrow while their progeny must rapidly differentiate to myeloid (blood cells—erythrocytes, neutrophils, basophils, eosinophils, monocytes, macrophages, thrombocytes (platelets)) and lymphoid (immune cells—T-, B-, NK-, dendritic cells) cell types. Skin and intestines are living barriers exposed to the environment and must be constantly renewed to maintain their structural organization. The location of stem cells in skin and intestine is an important part of the structural organization of these tissues. Other tissues such as the skeletal muscle, bone, and neural tissues have low turnover and long-lived stem cells. It is difficult to identify cycling stem cells in these tissues, and it often takes an injury event to see the stem cell activity.

2.8 Cell death is normal—apoptosis, autophagy, necrosis, and necroptosis

What counterbalances cell proliferation? Most cells in the body encounter neighboring cells and become space limited. This **contact inhibition** leads proliferating cells to slow the rate of mitosis and then stop dividing. Most adult cells in the body are quiescent and busy performing their function within their tissue and organ system. There are also important cell processes that help control cell proliferation that are active in early tissue formation, but also important to eliminate damaged cells, or cells that become diseased. These processes include apoptosis, autophagy, necrosis, and necroptosis, although further classification is possible. Each of these processes can be characterized morphologically and biochemically by the expression of certain genes and proteins. These dynamic, normal processes

can be encountered in the development of tissue engineered composite grafts consisting of cells and biomaterials.

Apoptosis is a natural process and is also called programmed cell death. In programmed cell death, the cell is said to die from within and the nuclear membrane will breakdown and the nucleus and DNA vesiculate within the cell, while the cell membrane is still intact (Fig. 2.5). Eventually, the cell plasma membrane breaks down and the cell contents are released. One of the natural reasons for apoptosis is a cell “recognizes” it is not in the right spot. For example, during development, apoptosis is part of the

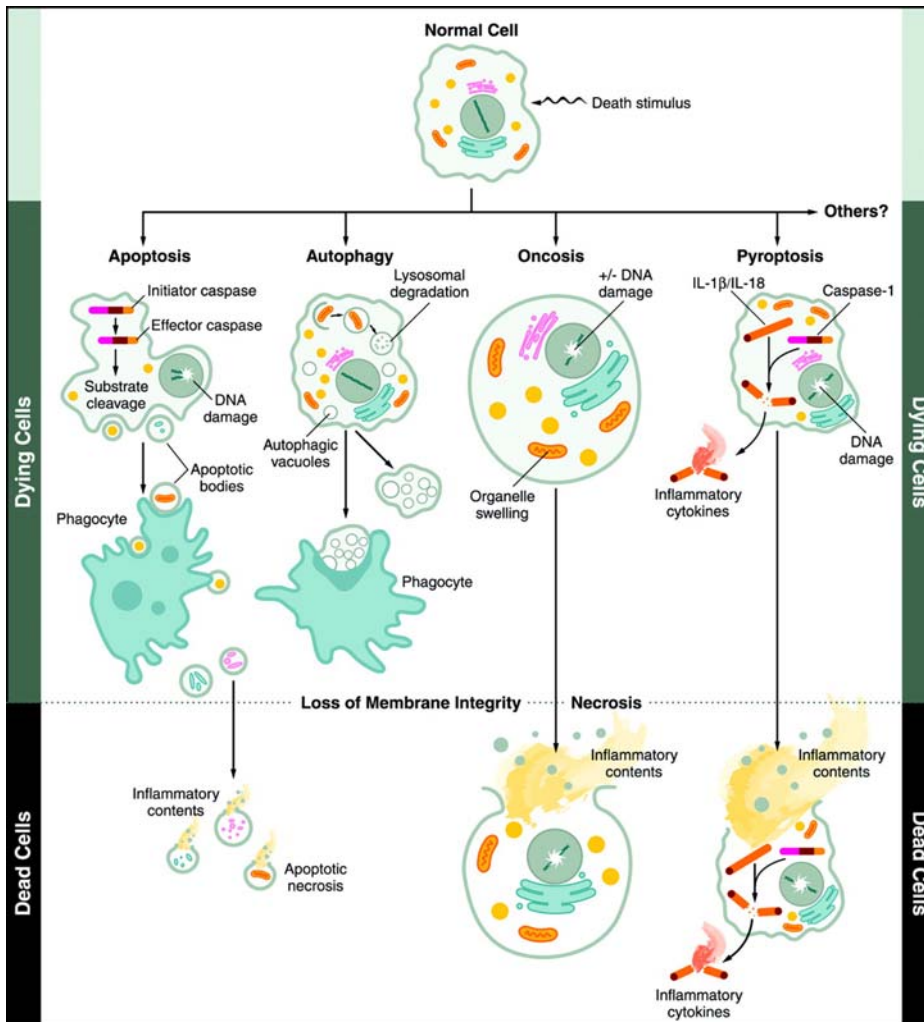


FIGURE 2.5

Schematic shows how a normal cell may receive signals that trigger cell death by several controlled biochemical pathways resulting in apoptosis, autophagy, necroptosis (which include oncosis and pyroptosis), or the uncontrolled disruptive necrosis pathway.

process of morphological change and many cells, such as the cells of the web between the developing fingers, may die a natural death from apoptosis.

A series of suicidal enzymes are involved in apoptosis and the triggering of caspase activity, particularly caspase 3, causes a cascade that is hard to reverse. Anoikis is a form of apoptosis that occurs when cells are not attached to a surface or matrix. That is, many cell types require attachment for normal survival. The signals that initiate apoptosis are not all understood, but the tissue engineer should recognize the occurrence of this natural process. It counterbalances proliferation that might otherwise proceed unchecked. There is also a potential benefit from dying cells where they can provide signals, vesicles, and subcellular components to the surrounding cells. Therefore, the natural process of dying cells is an important part of the normal tissue homeostasis.

Autophagy or “self-eating” is another cell intrinsic process that reduces cell number. It is characterized morphologically when the intracellular membranes become meshed with the lysosomal membrane and the contents are dissolved by intrinsic enzymes. During times of cellular stress including nutrient deprivation or toxic agents, autophagy increases. The elimination of damage mitochondria, or mitophagy, occurs by this method. Biochemically, autophagy stimulated by withdrawal of growth factors or nutrients activates several gene products including those of the mammalian Target of Rapamycin pathway and involves beclin 1 and phosphatidylinositol-3-kinase. Once the membrane bound autophagosome is formed, fusion with a lysosome leads to proteolytic destruction of the contents.

Necroptosis (Fig. 2.6) is form of natural programmed cell death that may be triggered by pathogens wherein a few tissue cells will die and stimulate a robust immune response that can limit the further pathogen effect and facilitate tissue recovery. It is the sacrifice of a few cells for the enhanced response to a pathogen. As an example, during the recent COVID-19 pandemic, patients with greater virus load recovered poorly thought to be due to necroptosis in the lungs, sometimes exacerbated with follow-on infections such as pneumonia. The signals that trigger necroptosis involve tumor necrosis factor α (TNF- α) and are different from the caspase cascade accompanying apoptosis. TNF- α binds to the TNF

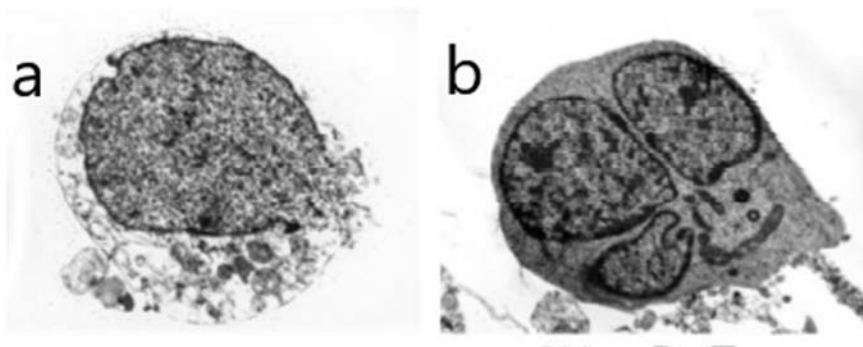


FIGURE 2.6

Electron micrographs of cell death occurring by apoptosis (a), necrosis (b), autophagy or necroptosis. From Fink, Cookson. *Infect Immun.* 2005;73:1907–1916.

receptor 1 and triggers TRADD expression and TRAF2 further signals the process of necrosome formation, aggregation of pore forming molecules at the plasma membrane, and to the release of damage-associated molecular proteins that cause immune stimulation. Pyroptosis is another form of lytic necroptosis but uses a different caspase to begin the process. Oncosis is characterized by the increased membrane permeability and swelling of mitochondria, leading to loss of homeostasis. Its cause may be the disruption of membrane pore complexes or viral infection where cell bursting spreads the virus to neighboring cells.

Necrosis (Fig. 2.6) is nonprogrammed cell death, wherein cells are starved for nutrients by lack of blood supply such as when the cell/tissue experiences trauma and the cells become damaged and die. Necrosis can also occur when there is extensive inflammation or swelling that disrupts the blood supply and tissue environment. The cellular debris will be cleared by macrophages attracted to the region by biochemical signals triggered by necrosis. Lack of nutrients can also lead to limits on tumor growth wherein the tumor center may become necrotic. In tissue engineering, necrosis is one of the major factors limiting the size of a cell/tissue construct as the nutrient supply and the elimination of waste products requires a vascular network and remains a challenge.

These natural cell loss processes are another consideration for the tissue engineer, and steps to analyze and understand if the loss of the cell component of a tissue construct is occurring by one of these processes are important. The mode of failure is always a design consideration in the early development of new composite constructs, biomedical devices, and medical therapies. There are biochemical agents that can interrupt and limit the pathways of apoptosis, autophagy, necrosis, and necroptosis if they occur, and this may lead to design criteria changes.

2.9 Characterization of stem cells—protein expression

Stem cell proliferation and their differentiation into functional cell types are usually mutually exclusive because the cell DNA must be organized into tightly packed chromosomes prior to cell division and this compact state of DNA is not compatible with the expression of the many genes needed for the differentiated phenotype. Trial and error studies have identified common characteristics of each stem cell type from different tissue sources that allow purification, classification, and identity criteria. Several useful techniques analyze the proteins expressed by stem cells using antibodies or agents specific to known proteins, either on whole cells using specific antibodies and fluorescence microscopy or on extracts of cell proteins separated by polyacrylamide gel electrophoresis and transferred to a membrane and probed using antibodies (western blotting). More machine intensive methods may use gas chromatography coupled to mass spectrometry to analyze protein expression, or the antibody-based flow cytometry that gives a snapshot of the proteins and therefore the genes expressed by the cell. Flow cytometry and the related technique **fluorescence activated cell sorting (FACS)** examines the surface phenotype of the cell by using antibodies that bind to the proteins on the cells of interest. In sorting mode, particular subsets of a cell population can be isolated for further study. Each stem cell type expresses a characteristic spectrum of surface markers that allow them to interact and absorb information from their

environment. The flow cytometry characterization of stem cells is usually performed during log phase growth with most of the cells in the G₁ state of the cell cycle although there may be some cells at all cell cycle stages in the sample. If the FACS machines are sophisticated enough, it is possible to analyze 10,000 cells in minutes and the large number of data points allows for strong statistical confidence (Fig. 2.7). There are ~300 standardized cell markers identified using monoclonal antibodies designated as “cluster of differentiation” CD antibodies. Many CD markers are used to characterize stem cells and multipotential cells although positive reaction with the antibodies is not conclusive evidence of

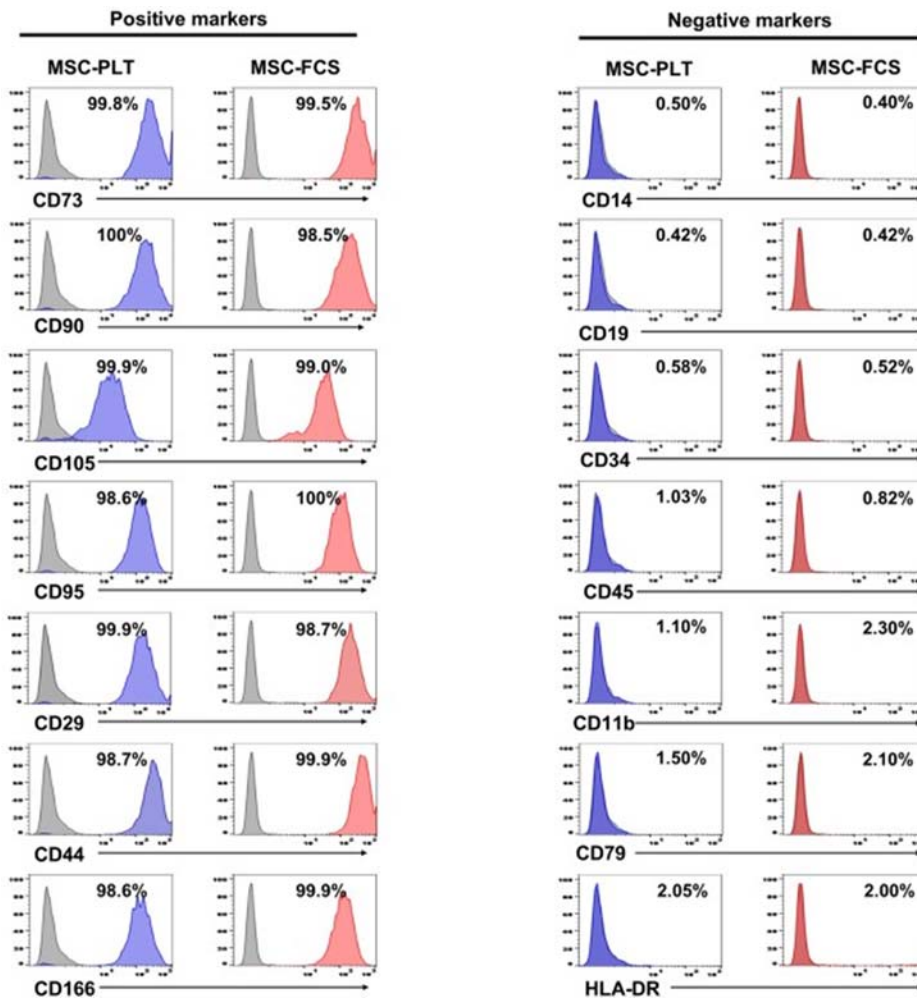


FIGURE 2.7

FACS identifies cells (here MSCs grown in PLT (platelet lysate) or FCS (fetal calf serum)) that bind positively (greater than 99%) with antibodies against surface molecules on cells versus those that do not bind (less than 2.5%) to MSCs. *From Reis et al. Global phenotypic characterization of human platelet lysate expanded MSCs by high-throughput flow cytometry. Sci Rep. 2018;8: 1–12.*

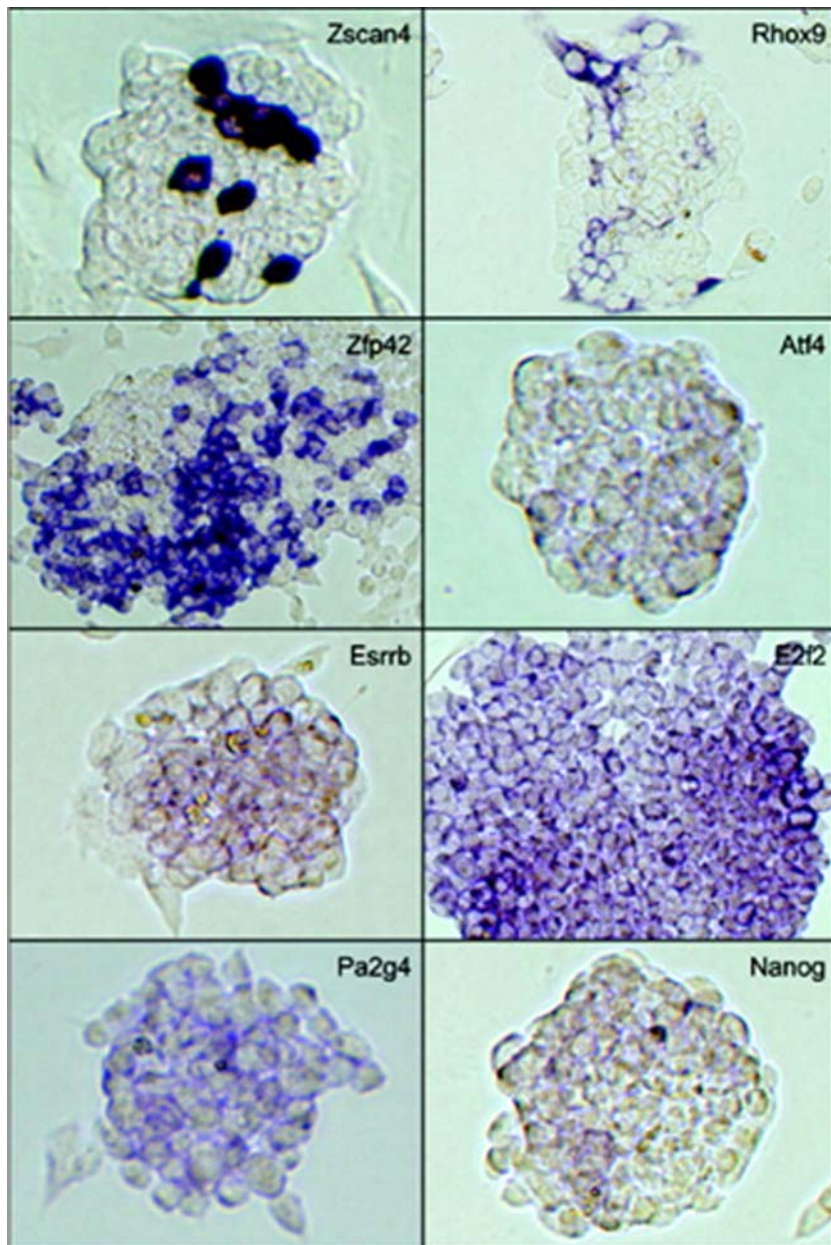
“stemness.” While a positive reaction with some markers is essential for identifying a type of stem cell, equally important is the negative reaction or lack of expression of other markers. In Fig. 2.7, the FACS analysis demonstrates the cells are negative for hematopoietic cell markers CD45, CD14, CD34, and the HLA II protein (A, B, C, I) and positive for expression of surface markers of mesenchymal cells CD105, CD166, CD44, CD29 (D, E, F, G), while weakly positive for the HLA I surface protein (H). The dotted line is the signal for nonspecific antibody isotype control in each binding experiment, the x axis is a log scale of the signal intensity, and the y axis records the number of positive cells at each intensity.

Extensive analysis of each type of stem cell also reveals microheterogeneity within the stem cell population. That is, seemingly homogeneous colonies of stem cells in a culture dish can be examined with protein or RNA-specific probes to show neighboring colonies and neighboring cells within each colony may have very different characteristics beyond growing together on the same dish. In Fig. 2.8, mouse ESC line 129.3 were cultured to ~ 100 cell-size colonies, fixed, and then probed with complimentary gene probes for RNAs of interest such as Nanog, Zscan4, Rhox9, etc. The blue stained cells express the RNA of interest and show that even within a colony derived from a single cell, heterogeneity has arisen without outside direction. This may be described as temporal stochastic differentiation or **temporal stochasticity** wherein a seemingly stochastic differentiation event in one cell may lead to propagation of gene expression changes in neighboring cells, and this may be followed by stepwise hierarchal differentiation within the colony. That is, the hierarchal levels of committed differentiation become available only after a stochastic differentiation event has occurred.

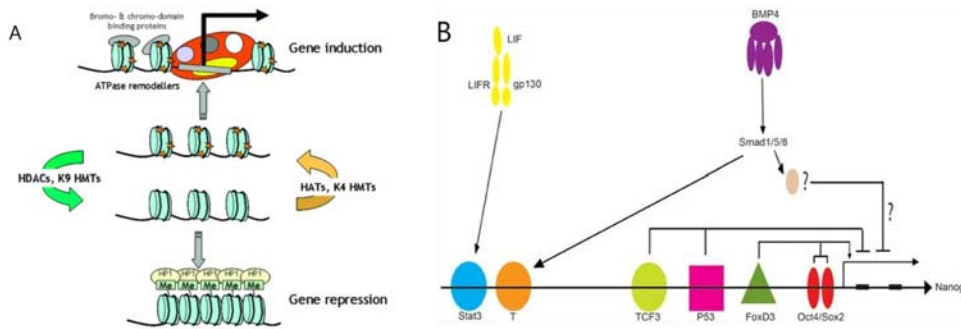
2.10 Characterization of stem cells—RNA analysis by RT-PCR, microarray, and RNA-sequencing

Another technique that is complimentary to flow cytometry and is widely employed in the study of stem cells and other cells is gene expression analysis, usually in the form of RNA analysis by the reverse transcription-polymerase chain reaction (RT-PCR), microarray, or direct RNA sequencing (RNA-seq). Kerri Mullis was awarded the 1993 Nobel Prize in Chemistry for demonstrating the amplification of specific RNA sequences expressed in cells using viral reverse transcriptase, followed by DNA polymerase reactions for multiple rounds (the “chain reaction”) followed by electrophoretic separation. These methods are now performed by machines capable of quantifying the level of gene expression activity by the specific RNA level. The RNA expression is exquisitely sensitive to the state of the cell and is one of the best measures of a cell’s identity, be it a stem cell or adult cell. RNA transcription is dependent on the degree of accessibility of transcription factors to genes and this is related to the compaction of DNA into nucleosomes and DNA modifications.

In Fig. 2.9a, the DNA nucleosome compaction and methylation modifications are depicted. These **epigenetic modifications of DNA** can alter gene expression, RNA transcription, and protein expression and are characteristic of individual cell types. These stable DNA modifications are not encoded in the A-T-C-G sequence and include chromatin structure remodeling, DNA methylation of guanine, and

**FIGURE 2.8**

Single colonies of stem cells may show differences in gene expression even among recent daughter cells within a single colony. Here each group of cells was probed with an RNA probe for the gene shown in the upper right of each pane. *From Carter et al. Gene expression patterns. 2008;8:181–198.*

**FIGURE 2.9**

Targeted DNA modifications at gene regulatory regions (a) under the control of histone methylases (HMTs), histone acetyltransferases (HATs), and histone deacetylases (HDACs). (b) the nuclear cell signaling events that must occur to turn on transcription of the stem cell Nanog gene is depicted. *From Adcock IA, Ford P, Ito K, Barnes PJ. Respir Res. 2006;7:21.*

histone protein modifications. There are also genes that are noncoding RNAs. RNA-seq can also routinely examine the long noncoding RNAs as well as regulatory micro-RNAs, neither of which are translated into protein. The DNA structure that contains bromo- and chromo-domains permits recruitment of ATP-dependent chromatin remodeling factors to open promoters and allow further recruitment of the basal transcription machinery. Deacetylation, frequently followed by histone methylation, establishes a base for highly repressive structures, such as heterochromatin. Acetylated histone tails are shown as yellow stars. Methylation (Me) is shown to recruit heterochromatin protein 1. In Fig. 2.9b, the internal cell signaling events that must occur to turn on transcription of the stem cell Nanog gene are depicted. Interaction of the soluble leukemia inhibitory factor and bone morphogenic factor 4 with their surface receptors stimulates second messengers STAT3 and Smads that bind to upstream DNA sequences, opening additional binding sites for transcription factors Fox D3 and Oct 4/Sox2 that allow the transcription machinery to transcribe the Nanog gene. Often for differentiation to proceed, Nanog must be silenced by negative regulators Tcf3 or p53 binding to their DNA regulatory sequences.

During analysis, the mRNA expressed by the cells is first isolated to eliminate any contamination from DNA. For RT-PCR, the target gene of interest is known, and specific primers are used to amplify the sequence so that its abundance is relative to the number of cycles of amplification. Even minor RNAs become abundant and can be analyzed and compared to levels of other RNAs or the levels in other cell types. Microarray analysis starts with the same mRNA isolation and reverse transcription, but then hybridizes the mixture of complementary DNA to known samples of DNA representing multiple genes of interest, anywhere from 40 to 200 or more, that then can be quantitatively analyzed by array imaging methods. In general, this is more than enough comparisons for most studies. But, perhaps no method in cell biology creates more data per analysis point than RNA-Seq. RNA-seq analyzes all RNAs. Short random primers or linkers that serve as universal primers allow all RNA species

to be sequenced. This includes mRNA, noncoding RNA, RNA splice variants, regulatory micro-RNAs, and very low abundance RNAs. The advantages of RNA-seq are many, but the sequencing requirements, storage, and data analysis make it more expensive and require more dedicated time and effort. The newest sequencing machines are designed to have either (1) high throughput, (2) long reads, or (3) nanopore direct read capabilities (see **RNA-Seq methods compared**). Notably, these sequencing techniques are also used for sequencing DNA, and in May 2021, the final segments of the human genome were finally completed, using the long read techniques 2 and 3. Even though there were early claims of this accomplishment in 2003, it has taken another 18 years of dogged determination to complete the difficult segments of repetitive G-C-rich DNA associated with the centromeres, and several new genes have been found there.

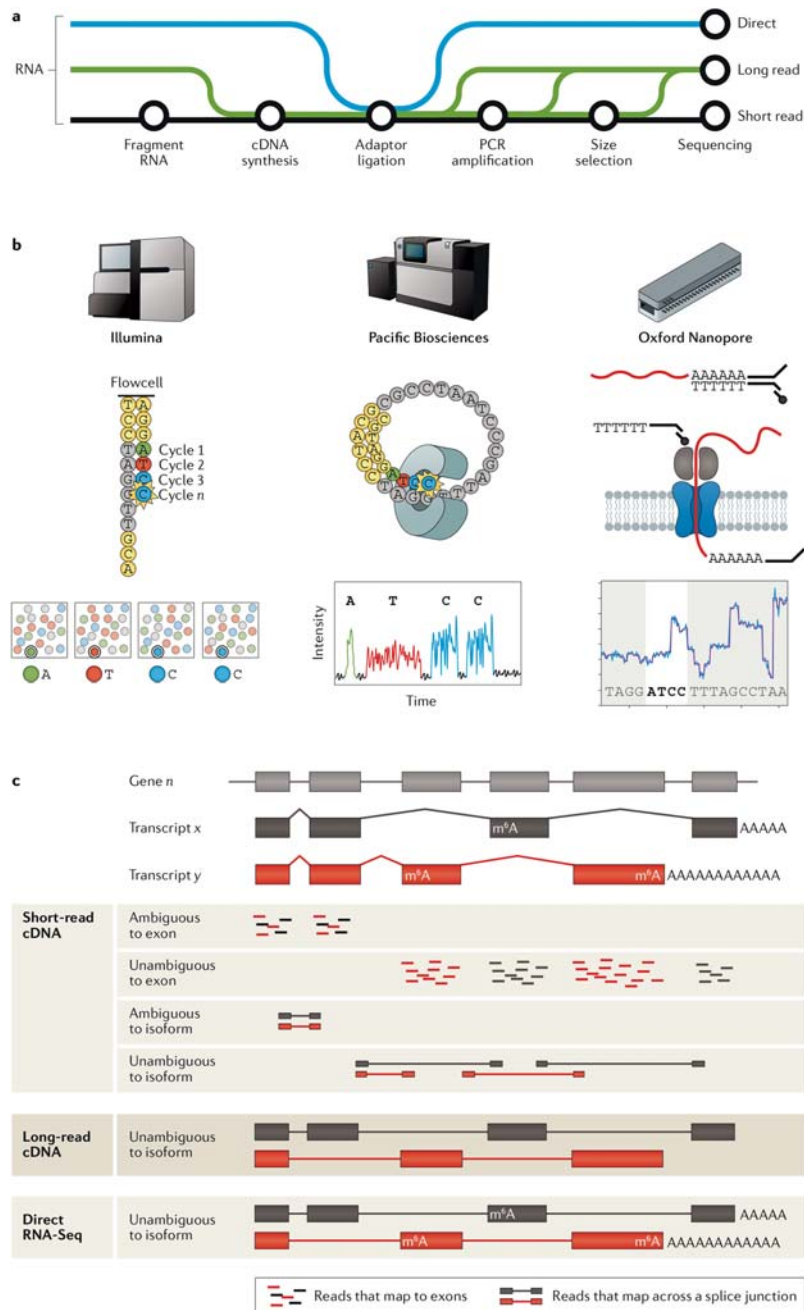
RNA-Seq methods compared

Shown in Fig. 2.10 is an overview of library preparation methods for different RNA-seq methods, which can be categorized as short-read sequencing (black), long-read complementary DNA [cDNA] sequencing (green), or long-read direct RNA-seq (blue) (Fig. 2.10a). The complexity and bias of library preparation varies according to the specific approach used. The short-read and long-read cDNA methods share many of the same steps in their protocols, but all methods require an adaptor ligation step, and all are affected by sample quality and computational issues upstream and downstream of library preparation. An overview is shown of the three main sequencing technologies for RNA-seq (Fig. 2.10b). The Illumina workflow (left panel): after library preparation, individual cDNA molecules are clustered on a flow cell for sequencing by synthesis using 3' blocked fluorescently labeled nucleotides. In each round of sequencing, the growing DNA strand is imaged to detect which of the four fluorophores has been incorporated and reads of 50–500 bp can be generated. The Pacific Biosciences workflow (middle panel): after library preparation, individual molecules are loaded into a sequencing chip, where they bind to a polymerase immobilized at the bottom of a nanowell. As each of the fluorescently labeled nucleotides is incorporated into the growing strand, they fluoresce and are detected, and reads of up to 50 kb can be generated. The Oxford Nanopore workflow (right panel): after library preparation, individual molecules are loaded into a flow cell, where motor proteins, which are attached during adaptor ligation, dock with nanopores. The motor protein controls the translocation of the RNA strand through the

nanopore, causing a change in current that is processed to generate sequencing reads of 1–10 kb. Comparison of short-read, long-read and direct RNAseq analysis can be seen in Fig. 2.10c. Over 90% of human genes (gene *n*) are alternatively spliced to form two or more distinct and expressed isoforms (transcripts *x* and *y*). The complexity of information captured increases from short-read cDNA sequencing, where isoform detection can be compromised by reads that cannot be mapped unambiguously, to long-read methods that directly sequence isoforms. In short-read cDNA sequencing, a significant proportion of reads map ambiguously when an exon is shared between isoforms; reads that span exon–exon junctions can be used to improve the isoform analysis but can also be mapped ambiguously when a junction is shared between isoforms. These issues complicate analysis and the interpretation of results. Long-read cDNA methods can generate full-length isoform reads that remove, or substantially reduce, these artifacts and improve differential isoform expression analysis. However, these methods rely on cDNA conversion, which removes information about RNA base modifications and can only make crude estimates of polyadenylation (poly(A)) tail length. Direct RNA-seq enables full-length isoform analysis, base modification detection (such as N⁶-methyladenosine [m⁶A]), and poly(A) tail length estimation.

Stark, R. et al. RNA sequencing: the teenage years. *Nat Rev Genet* 20, 631–656, 2019, <https://doi.org/10.1038/s41576-019-0150-2>.

Continued



Overview of library preparation methods for different RNA sequencing (RNA-seq) methods. From Stark R, et al. *RNA sequencing: the teenage years*. Nat Rev Genet. 2019;20:631–656. <https://doi.org/10.1038/s41576-019-0150-2>.

2.11 Characterization of stem cells—cell differentiation

The proteins and RNAs expressed by stem cells are a good starting point for characterization, but the differentiated cell types are quite different from their originating stem cells and multiple differentiation pathways make the eventual cell type unclear. Differentiation of stem cells produces the variety and diversity of cell types seen in the body, about 230 different types of cells. Of these, our current knowledge only allows us to direct the production of ~ 30 cell types reproducibly in small amounts, millions to a few billion cells. In the case of somatic cell nuclear transfer (see Classical Experiment) wherein a somatic nucleus is placed into the enucleated embryonic cell, the “new” created cell is then implanted in the uterus of a surrogate mother, where further development and birth of a whole organism is the final test. If the differentiation potential of the cells under study is thought to be only pluripotent, the differentiation potential of the stem cells may be studied *in vivo* by implanting the cells of interest into an immune incompetent mouse or rat host, where the cells will not be immune rejected, and differentiate *in vivo*. To test the differentiation of stem cells *in vitro*, assays have been developed that predict the *in vivo* performance of transplanted cells. Ideally, this assay is rapid, quantitative, inexpensive, and a direct measure of one or more desirable traits. However, differentiation is a cellular process that occurs over days with many changes in gene expression as tissue-specific transcription factors are produced and inhibitors are eliminated. Early differentiation is usually evident in 2–3 days such as the production of the extracellular protein aggrecan by MSCs undergoing chondrogenic differentiation, but the mature chondrocyte producing type II collagen in abundance may take 2–3 weeks to produce. Further, the structural organization and morphology within a group of differentiating cells should resemble their *in vivo* mature counterparts and this may take days to weeks to be evident. Cellular differentiation assays are used during the development of therapeutic products and the tissue engineer should be familiar with them. The later sections of this chapter and many of the chapters in this book describe such assays and their development, and the detailed protocols are available in the literature.

2.12 Stem cell signaling—the Wnt and β -catenin pathway

How do stem cells know what to do, and what to do next? Stem cells may have intrinsic differentiation potential, but stem cells respond to cues in their environment that allow the next steps to happen. That is, proliferation and differentiation are highly controlled through input from surrounding cells and environmental signals and require controlled energy expenditure that is highly regulated by available ATP, GTP, etc. The closer one looks, the more evidence there is that stem cell pools and their proliferation and differentiation are tightly regulated by signals from their environment and neighboring cells.

Not all mechanisms of stem cell signaling and control are understood but a few are. Cells have surface receptors that can bind to extracellular ligands, and this triggers a cascade of intracellular biochemical events that results in changes of gene expression that allow the next differentiation (or proliferation, or cell death) events to occur. One of the better understood models of control of gene expression is the Wnt/ β -catenin pathway. Wnt is a secreted molecule that binds to surface receptors on another cell and triggers intracellular β -catenin activation and downstream events (see Clevers et al. 2014). The intracellular β -catenin level is usually low as it is bound by inhibitors and degraded via the ubiquitination/proteasomal pathway. Here we present a simplified version of Wnt signaling, but there are several surface receptors or coreceptors that can trigger changes in Wnt gene expression. Wnt was originally identified as integration site 1 or Int-1, an insertion site for mouse mammary tumor virus that could

cause breast tumors in mice and sequencing of this proto-oncogene identified it as a secreted protein with homologs across nearly all animal species, vertebrates, and invertebrates. In *Drosophila*, mutations in this gene led to embryo segment and wingless mutants. Wnt is a three letter contraction of wingless/int-1. In *Xenopus*, microinjection of the Wnt mRNA led to duplication in body axis indicating a strong influence in early development. Several gene products that could cause axis duplication were identified suggesting a family of related genes and developmental pathways. While Wnt genes are active in early development, they are also found to be expressed in self-renewing tissues including skin, intestine, bone, and blood. Identifying which genes are active with which pathways, and their control mechanisms across species, has been a 20+ year endeavor. Genome sequencing has found the Wnt-related homologs and in mammals there are 19 Wnts. The Wnt proteins are secreted, but they are not very soluble due to lipid modifications, so they signal over short distances to neighboring cells. However, secreted Wnts may be bound by lipoprotein particles to form multimers that are more soluble and act to create broader gradients away from the originating cell(s). Wnts are utilized by most if not all stem cell populations and serve here as a model for stem/progenitor cell control of proliferation and differentiation.

When Wnt binds to its cell surface receptor, it activates cytoplasmic β -catenin, a central molecule in intracellular signaling (Fig. 2.11). In the left panel, Wnt signaling is off and cytoplasmic free β -catenin levels are kept low by binding proteins such as APC/Axin2/GSK3 β that sequester β -catenin for ubiquitination and degradation. In the right panel, when extracellular Wnt levels are high, the co-receptors frizzled, Lrp5/6 associates and causes β -catenin to be released from the destruction complex. The intracellular levels of β -catenin rise and it accumulates in the nucleus which activates gene transcription. But the Wnts may also signal through the noncanonical pathways bypassing β -catenin and utilizing planar cell polarity or Ca²⁺ pathways to activate gene families such as Jun and NFAT. β -catenin is

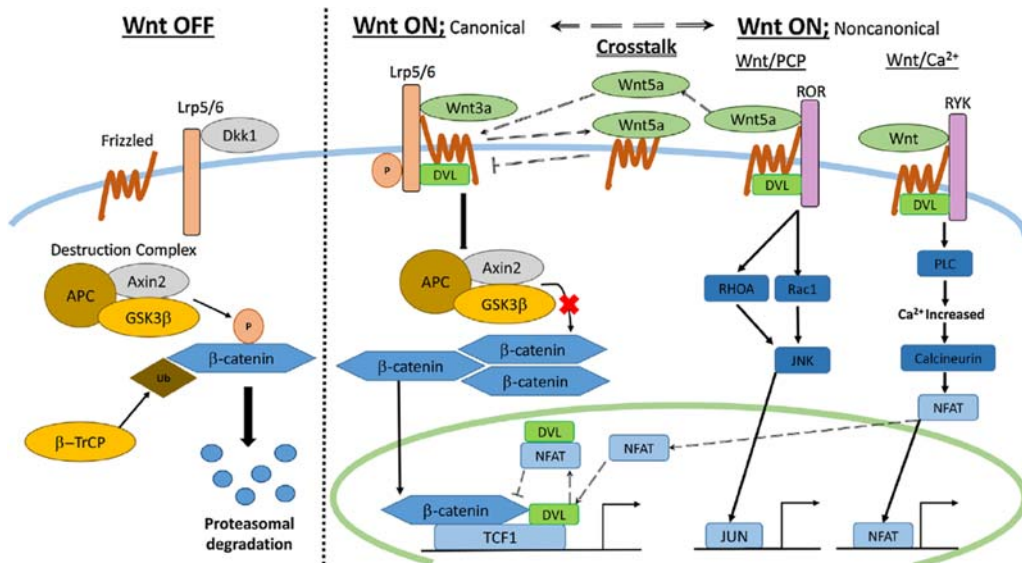


FIGURE 2.11

Wnt pathways. From Nagano. Jap Dental Sci Rev. 2019 55:80–87.

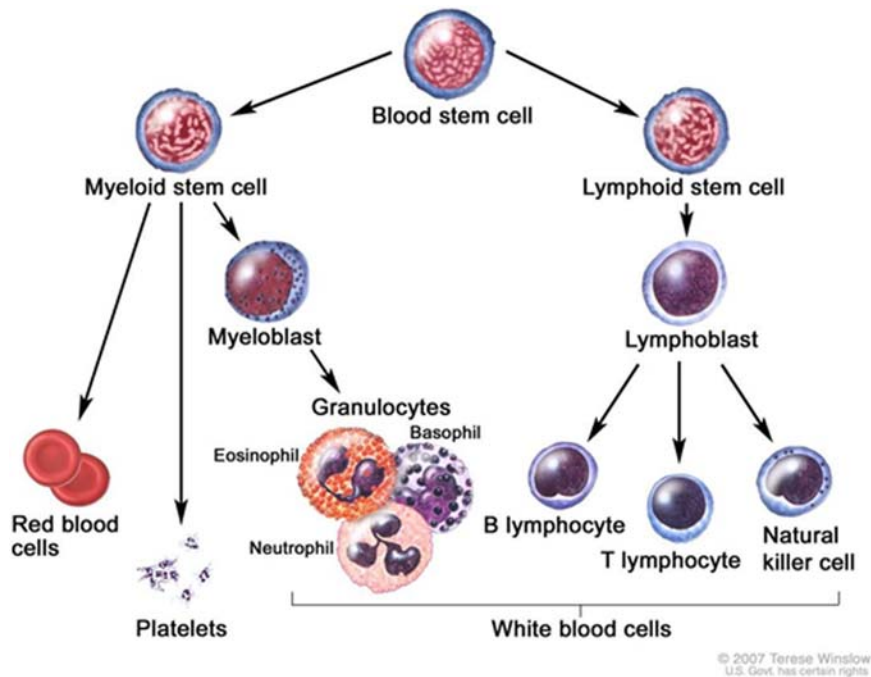
normally phosphorylated and ubiquitin ligase causes its rapid turn-over in the cell cytoplasm, but when Wnt binds its cell receptor, phosphorylation of β -catenin is blocked, and it accumulates in the cytoplasm (see Chapter 4 Cell signaling). This accumulation of β -catenin results in its translocation to the nucleus where it binds gene repressors, weakening their association with the DNA, and allowing new expression from a wide number of genes. In this way, Wnt-triggered accumulation of β -catenin can result in cell proliferation or terminal differentiation, or both events may occur in a tissue where regeneration and differentiation are occurring in near proximity such as the intestinal crypt and hair follicle. The forgoing describes the common canonical Wnt/ β -catenin pathway, but noncanonical Wnt pathways are also recognized that are not β -catenin dependent.

What initiates intracellular Wnt signaling? There are several surface receptors that bind extracellular Wnt, and some have coreceptors that intensify or mitigate the signaling effects. There are seven-pass membrane receptors such as the Frizzled gene family of 10 members in man, and the single-pass receptors represented by the R-spondin family of 4 genes, and the low density lipoprotein-related protein coreceptor (LRP, mostly LRP5/6) family. The RYK and ROR receptors are coreceptors of Frizzled and can be triggered in the absence of Wnts to activate tyrosine kinase within the cell to alter gene expression. These receptors and coreceptors trigger intracellular signaling. Many of these receptor families were given their names from the names of *D. melanogaster*, *C. elegans*, or *X. laevis* mutants that were responsible for their initial identification and function, prior to their identification in man.

2.13 Hematopoietic stem cells

HSCs were the first human somatic stem cells identified and isolated. From before birth to the time of death, the blood cells are continually replaced. HSCs are the precursor cells for all the blood lineage cells including the lymphoid PCs that differentiate to the cells of the immune system such as T cells, B cells, and natural killer (NK) cells, as well as the myeloid PCs that differentiate to monocytes/macrophages, basophils, eosinophils, red cells, and platelet releasing megakaryocytes. The importance of HSCs to the study of stem cells cannot be overstated as many of the stem cell concepts and definitions derive from the scientific and medical study of these stem cells. The first successful treatment of a leukemia patient with a bone marrow transplant was in 1956 and the donor was an identical twin, so there was no immune rejection. The *in vitro* study of HSCs really began in the 1960s with the experiments of James Till and Ernest McCulloch who studied the effects of radiation on bone marrow in mice at the Ontario Cancer Center in Toronto. They were able to develop methods to save animals from the effects of radiation by transplanting donor bone marrow that repopulated all the blood lineages. Through innumerable studies, the blood cell lineages have been traced from the adult cell types back to the blood stem cell—the HSC (Fig. 2.12). The gene expression mechanisms involved in lineage progression are understood. However, this process is inefficient in the lab and *in vivo* transplantation of immunologically matched HSCs is the usual course of therapy where initial engraftment of HSCs is the limiting event in successful transplantation. Many studies have allowed the relationships of HSCs and their progeny to be understood at a cell and molecular level, and the genes and signals involved in stepping from stem cell to adult differentiated cells are known. Still, there are many process details to be studied further.

The use of isolated human HSCs in transplantation studies is extensive and today HSCs are used for a diverse group of diseases and conditions including leukemia, lymphoma, inborn errors of metabolism, etc. Over 50,000 bone marrow or HSC transplantations are performed annually worldwide. HSCs, however, are difficult to grow in culture and much effort has been expended to perfect the HSC

**FIGURE 2.12**

Hematopoietic stem cells from bone marrow regenerate all of the adult blood cell lineages. From NIH website. [www.NIH.gov/hematopoietic stem cells](http://www.NIH.gov/hematopoietic-stem-cells).

in vitro expansion. The current challenges are many but in vitro expansions that can maintain the stem cell phenotype, along with reliable engraftment in patients needing a bone marrow transplant, are primary areas of research effort.

HSCs can be isolated from harvested bone marrow, but since this is an invasive procedure, it has become increasingly infrequent. Today, clinical mobilization of HSCs from the bone marrow is accomplished by treating the donor with the HSC growth factor granulocyte-colony stimulating factor (G-CSF) for several days which results in more circulating HSCs in the peripheral blood, and collecting the HSCs by the process of plasmapheresis, wherein the cells are collected for donation and the plasma fluid fraction, is returned to the donor. While the G-CSF induces the HSCs to proliferate in the bone marrow, they are partly held in the stem cell niche by HSC-stromal cell interactions of their surface receptors, notably stromal cell derived factor-1 (SDF-1) and its receptor on the HSC, chemokine receptor CXCR4. To help displace the proliferating HSCs from their bone marrow niche, a compound such as AMD3100 may be given; this compound interferes with the SDF-1/CXCR4 interaction and allows greater release of HSCs from bone marrow into the circulation for collection.

From the tissue engineering perspective, there are a number of areas needing further study including (1) improvements in clinical HSC isolation, (2) in vitro propagation of HSCs, and (3) gene-modified HSCs for many inborn errors of metabolism conditions. Blood from the placenta and umbilical cord is an

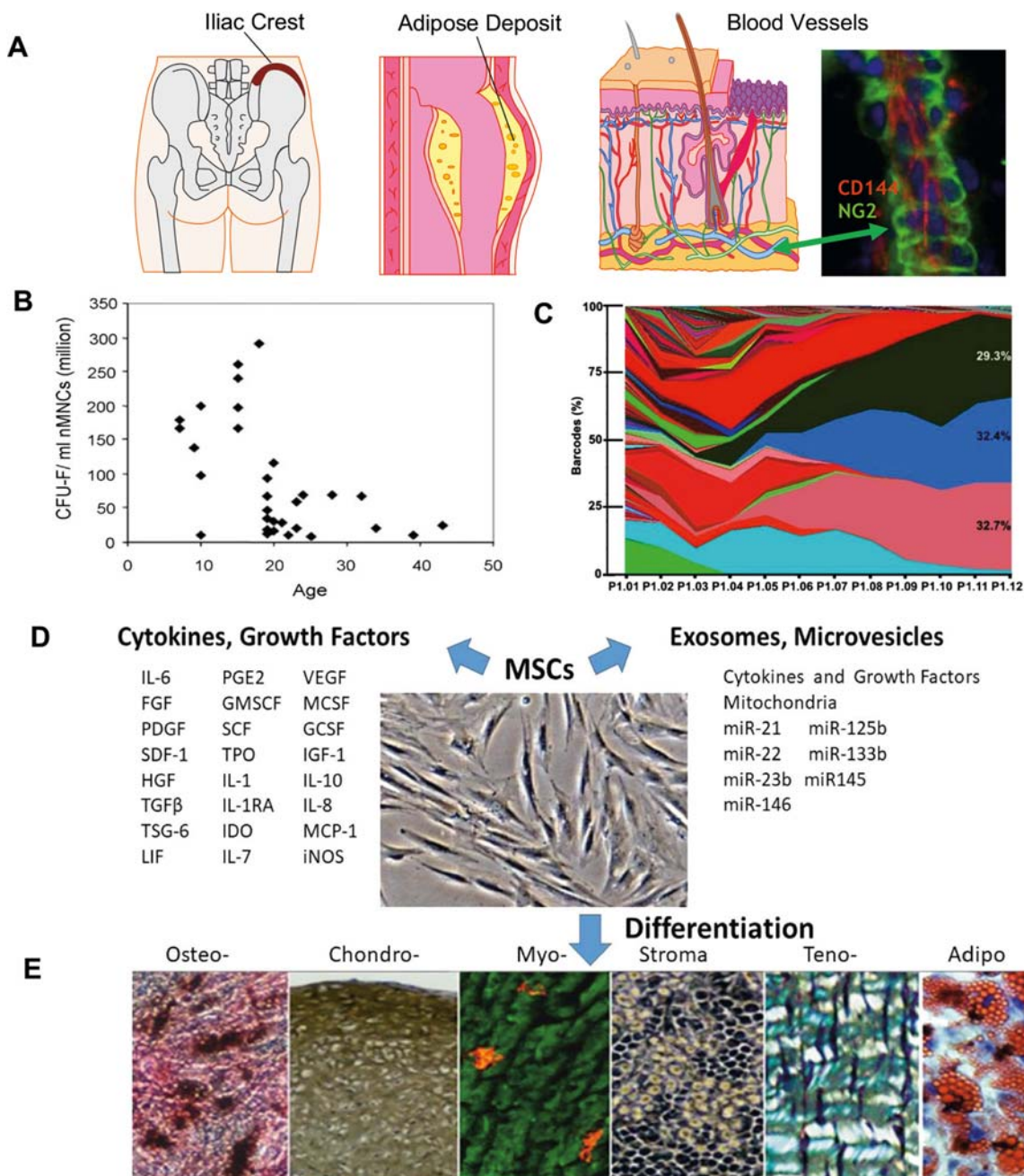
alternative rich source for hematopoietic stem cells. It is an appealing cell source because these tissues are usually discarded after birth, although as much blood as possible is usually drained from the umbilical cord to the new-born child. There is a Cord Blood Registry in most countries to continue to preserve and expand the use of this source of HSCs.

As of this writing in 2022, there were 4139 clinical studies utilizing HSCs that are completed, ongoing, or terminated listed at clinicaltrials.gov. Of these, 1355 are listed as recruiting, enrolling, or active for a diverse group of diseases or conditions including hematopoietic transplantation, thalassemia, sickle cell disease, metachromatic leukodystrophy, severe combined immunodeficiency, hematological malignancy and myelomas, and aging.

2.14 Mesenchymal stem cells

MSCs, also known as multipotential stromal cells and mesenchymal stromal cells, collectively “MSCs,” are a class of multipotent adult stem cells that can be isolated from many tissues but are most frequently isolated from bone marrow or adipose tissue (AT). It is common to designate MSCs by their tissue of origin such as BM-MSC for bone marrow-derived MSCs and AT-MSC (or MSC-Ad) for AT. The MSCs in bone marrow are rare and constitute only 0.001%–0.01% of the nucleated cells in bone marrow, so they are cultured *in vitro* to expand the cell numbers. For isolation of human MSCs, a bone marrow aspirate is taken from the iliac crest under a local anesthetic and the aspirated sample is subjected to density centrifugation and the layer containing the nucleated cells is cultured in nutrient medium. After 10–14 days in the incubator, colonies of proliferating cells are visible under the microscope. By 20–30 population doublings over 3–4 weeks, there are 100–300 million cells for study or medical use. MSCs have been shown to differentiate to several important lineages *in vitro* and *in vivo* such as bone, cartilage, and fat, with strong fidelity. That is, the vast majority of the MSCs differentiate without the appearance of other unwanted lineages, a problem that is common with pluripotent stem cells.

Many of the characteristics of MSCs are depicted in [Fig. 2.13](#). The figure depicts the tissue sources (A-B), the expansion of clonal populations (C), the growth factors expressed by, and the exosome contents produced by MSCs (D), and their differentiation to osteocytes, chondrocytes, myocytes, stroma, tenocytes, and adipocytes (E). The MSCs can be readily isolated from bone marrow and AT, but all tissues harbor MSC-like cells as part of the microvasculature ([Fig. 2.13a](#)). In [Fig. 2.13b](#) the number of MSCs, indicated here as colony-forming units (CFU-F), isolated from bone marrow declines after 15–20 years of age and continues to decrease. MSCs are culture-expanded to achieve high numbers for research or therapeutic use. However, as shown in [Fig. 2.3c](#), there is a decrease in the clonal complexity with increasing passage, but the effect of this process on MSC uses is unclear. In [Fig. 2.3d](#), the MSCs are known to produce a large number of soluble or vesicle-bound growth factors and cytokines, as well as micro-RNAs, that can signal to other cells and tissues. Shown in [Fig. 2.3e](#) are the results of *in vitro* differentiation of the culture expanded MSCs to multiple cell lineages under separate and specific *in vitro* conditions. Nearly all the MSCs present in the assay differentiate to the desired lineage without evidence of other lineages. The standard chondro-, osteo-, and adipo-differentiation conditions are widely used, but additional *in vitro* conditions promote smooth muscle and striated muscle gene expression; changing medium conditions can induce expression of cardiac and liver genes as well.

**FIGURE 2.13**

Characteristics of mesenchymal stem/stromal cells. From Pittenger et al. NPJ Regen Med. 2019. 4:22–37.

Once differentiated, the MSCs express virtually all the hallmark genes and proteins of the differentiated cell types. Currently, the more prominent MSC therapeutic uses take advantage of the MSC's production of growth factors and cytokines and the responsiveness of other interacting cells, such as cells of the immune system.

The early studies of MSCs show parallels with HSC studies. In 1970, hematologist Alexander Friedenstein isolated cells from guinea pig bone marrow that could be grown *in vitro* to form colonies of fibroblast-like cells that he termed **colony forming units-fibroblastic** or CFU-F. The cells were placed in sealed chambers with a dialysis membrane that allowed the inflow of nutrients, and the chambers were imbedded under the skin of host animals for several weeks. When excised and examined histologically, some of the chambers contained bone and cartilage, indicating differentiation of the implanted cells to these mesenchymal lineages. Similar work was conducted with rabbit bone marrow cells in the 1980s, a time when the hierarchy of blood lineages was now understood, and Maureen Owen proposed a similar lineage hierarchy diagram for bone marrow CFU-F cells. Arnold Caplan and Steve Haynesworth were the first to isolate multipotential mesenchymal cells from human bone marrow that could form bone, cartilage, and fat when ceramic cubes containing the cells were implanted in mice. Caplan coined the term MSCs to describe these cells in 1991. Pittenger and colleagues later demonstrated that individual human bone marrow cells could be clonally expanded and would produce the three specific lineages—bone, cartilage, and adipose—without differentiating to the other lineages under *in vitro* conditions. This meant there were now strong lines of evidence for two multipotential cells from bone marrow, the MSC, and the HSC.

The AT is highly vascularized and AT-MSC cells can be found along blood vessels in the population of microvascular **pericytes** that are contractile and aid in moving blood along small vessels. These pericytes have similar cell surface markers to BM-MSCs, and this indicates that all tissues have “MSCs” as part of their capillary network. This suggests peripheral MSCs are nearer than bone marrow, and would quickly respond to tissue injury, but experience indicates that the number of peripheral MSCs are inadequate to repair most injuries of adult tissues, where scarring and fibrosis are usually the result.

The AT-MSCs can be isolated following liposuction by a centrifugation procedure that separates them from fat, and the cells be used immediately as an uncharacterized but enriched fraction of PCs termed the stromal vascular fraction. This allows their isolation and reimplantation at the time of medical procedures without *in vitro* cultivation. Alternatively, the cell fraction can be placed in the incubator to obtain an expanded population of cells for characterization and experimental studies.

Studies on MSCs were very few over 1970–1995 but expanded greatly with the isolation of human MSCs. Now over 60,000 papers have been published in the last 25 years, most using BM-MSCs or AT-MSCs. MSCs can be isolated from a bone marrow aspirate or AT and expanded over 20–30 population doublings and provide enough cells for most regenerative medicine purposes. However, MSCs cannot be propagated in an unlimited fashion *in vitro* at present. Whether this is due to an intrinsic limitation in the MSC lifespan or due to a limitation in the present *in vitro* culture conditions is not clear.

2.14.1 MSC modulation of the immune system

One of the surprising features of MSCs and perhaps one of the most useful is their ability to modulate immune responses and inflammation. When blood lymphocytes, which contain many T cells, are

mixed with cells from another individual, there is a burst of proliferation of the T cells as they respond to the foreign cells. This mixed lymphocyte reaction is a common test of compatibility and **immune suppression** of this response is necessary to avoid rejection anytime donor and recipient are not identical. **Autologous** (self) transplantation does not require immune suppression. However, MSCs do not stimulate **allogeneic** lymphocytes in vitro and are not rapidly rejected upon in vivo transplantation. MSCs can subdue or reduce the immune response to cells from a third party donor. After years of careful study, it is known that MSCs produce at least 11 factors that suppress lymphocyte activation. Each factor can play an important role in avoiding rejection, either from T cells, dendritic cells, NK cells, or B cells, and an increase in regulatory T cells is common as well (Fig. 2.14). Initial studies envisioned autologous use of human MSCs; however, studies with immune cells demonstrated that MSCs are not immediately rejected by immune cells, prompting the study of allogeneic MSCs in multiple therapies. The consequences of the interaction of MSCs with T cells (pathways 1 and 5), is a reduction in inflammatory T_{H1} and an increase in antiinflammatory T_{Reg} s and T_{H2} cells with the concomitant decrease in $IFN\gamma$, and increased levels of IL-10, IL-4, and IL-5. When MSCs interact with dendritic cells (pathways 2, 3, and 4), there is a decrease in proinflammatory mature DC1 with a decrease in $TNF-\alpha$ and IL-12, and an increase in antiinflammatory DC2 and immature DCs, with increased expression of suppressive IL-10. When MSCs interact with NK cells (pathway 6), there is a decrease in the expression of $IFN\gamma$. When macrophages interact with MSCs (pathway 7), there is a decrease in the proinflammatory M1

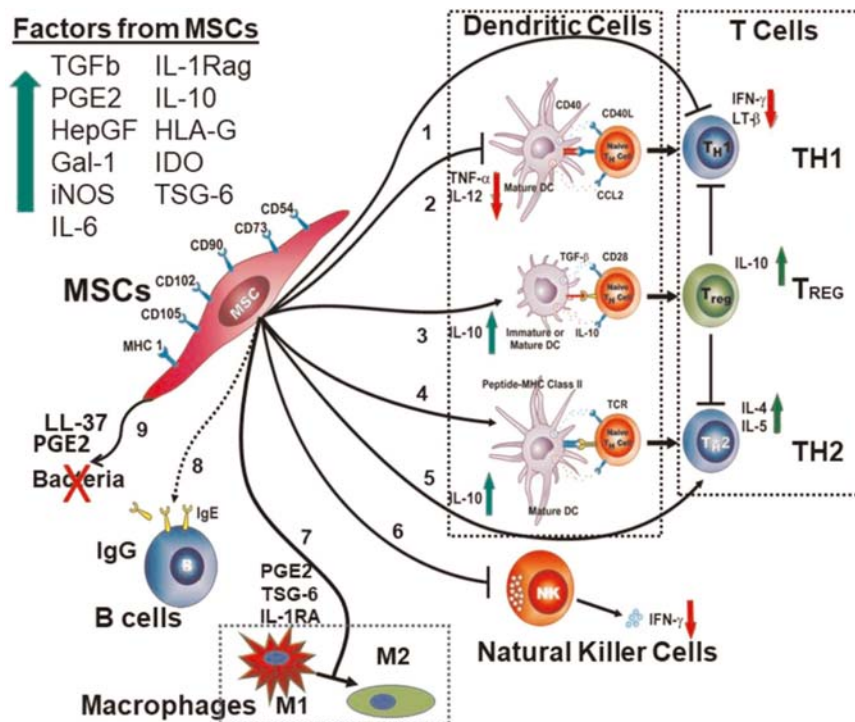


FIGURE 2.14

Immunomodulatory capacity of MSCs to escape rejection by immune cells. From Pittenger et al. NPJ Regen Med. 2019;4:22–37.

phenotype and an increase in the antiinflammatory M2 phenotype, with increased PGE₂, TSG-6, and IL-1RA. MSCs can also reduce the expression of secreted antibodies from B cells (pathway 8) and inhibit bacterial growth by a direct or indirect mechanism (pathway 9). All the reduction in the inflammatory response might suggest that greater levels of infection may accompany MSC delivery; however, this is not seen, and MSCs were found to produce factors that inhibit sepsis including PDE-2 and the antibacterial peptide LL-37.

The MSCs migrate to sites of injury, and it has become common to deliver MSCs intravenously for a variety of diverse disorders including heart ischemia, graft versus host disease, or pulmonary fibrosis. This homing to tissue injury effect has been widely studied and the release of factors at the site of injury, including SDF-1, has been evaluated as the inducer of MSC homing, although leaking from the capillaries at the site of tissue injury cannot be ruled out. Many studies have demonstrated that MSCs can increase angiogenesis, both in the infarcted heart and in models of peripheral artery disease. In summary, MSCs accumulate at the injury site, reduce local inflammation, release angiogenic factors and growth factors that accelerate injury repair, and thereby preserve neighboring healthy tissue.

At the time of this writing in 2022, there were over 1200 clinical trials listed at clinicaltrials.gov utilizing human MSCs from bone marrow or AT and 358 listed as recruiting, enrolling, or active in diverse medical conditions including cardiac infarction, acute respiratory distress, traumatic brain injury, osteoarthritis, bone nonunion, graft versus host disease, diabetes, aging frailty, and complications of COVID-19.

2.14.2 Epithelial stem cells—skin and intestine

The epithelia are specialized cell sheets composed of several cell layers that form boundaries between the body and the environment. Epithelial tissues, including the skin, stomach and intestinal lining, and lung and the air passageways, are exposed to the external environment and are tissues with the highest rates of cell turnover. Early in development, when epithelial tissues are expanding, the cell division axis is perpendicular to the tissue surface, but later as these “flat” tissues mature, the axis is rotated to provide upward cell replacement while maintaining the lower stem cell in its niche. The cells in epithelial tissues differentiate to a high level of diversification as well as remain in a state of near constant regeneration, although this slows with age.

2.15 Skin stem cells

The skin epithelium is exposed to the external environment which can be harsh in biological terms, being exposed to abrasion, temperature extremes, bacteria, environmental chemicals, and trauma injuries. The external skin makes up one of the largest organs and must regenerate constantly. The surface cornified epithelial layer is largely composed of dead cells that continually slough off, being replaced from the underlying layers of regenerating and maturing cells in a process that maintains the epithelial integrity for a lifetime. In the event of a wound to the skin epithelium, the activity of the skin stem cells (skSCs) is greatly accelerated to compensate. The skin is not uniform and has regions that vary in blood vessel density, pigments, glandular activity, hair density, sensory nerves, etc. So, there are many

challenges and opportunities for the tissue engineering of skin epithelium for wounds, diabetic ulcers, hair loss, and genetic diseases of the skin.

The outer interfollicular epidermis is replaced every few weeks and has histologically defined layers and the stem cells reside in the deepest layer (Fig. 2.15). Skin stem cells (SCs) in between the hair follicles are found in the deepest layers and regenerate the skin by either (left panel) the more orderly hierarchical model progressing through the TACs or the (right panel) stochastic model where the stem cell produces the dividing progenitor cells (PCs) that find their way to the epidermis as needed by the loss of surface cells. The skSC can divide to produce two stem cells, or a stem cell and a TAC, or 2 TA cells. The TA cells take on the role of assuring there are enough proliferating upwardly migrating cells to create the dermal fibroblastic layer (dermis) and the exterior cornified epithelial layer of dead cells (epidermis) that protects against moisture loss and the environment.

The hair follicles of skin add another level of complexity for regeneration and each hair follicle has an associated sebaceous gland. Hair follicles do not renew at the same constant rate as the skin interfollicular epidermis but proceed through periodic cycles of growth (anagen), resting (catagen), degeneration (telogen), and shedding (exogen). But the growing phase lasts 2–7 years while the other phases are months to days long. The structure of the hair follicle is very well studied, and the mature follicle has eight concentric cell layers identified by their position and the expressions of different keratin isoforms (Fig. 2.16). The hair follicles regenerate throughout life and their structure are complex. The hair has a regenerating segment with an outer and inner root sheaths and hair shaft that is periodically shed. A layer of TACs derive from the dermal papillae. Structures are often identified by the different keratin isoforms the cells express (K6, K7, K31, K32, K71, etc.). Following telogen, the hair regenerates from the deepest tip—the hair germ cells—but another group of stem cells reside in the bulge region adjacent to the sebaceous gland. In fact, cell labeling studies in mouse models have shown that if a certain group of hair follicle stem cells are ablated, other nearby cells can take over the missing cells role, thereby

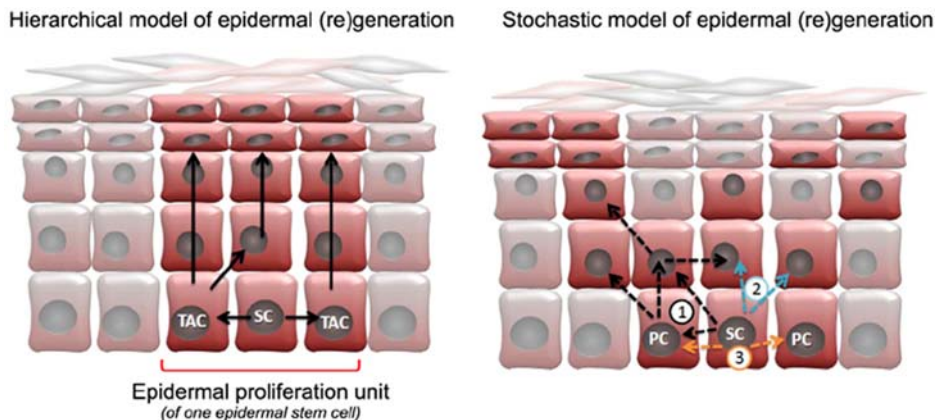
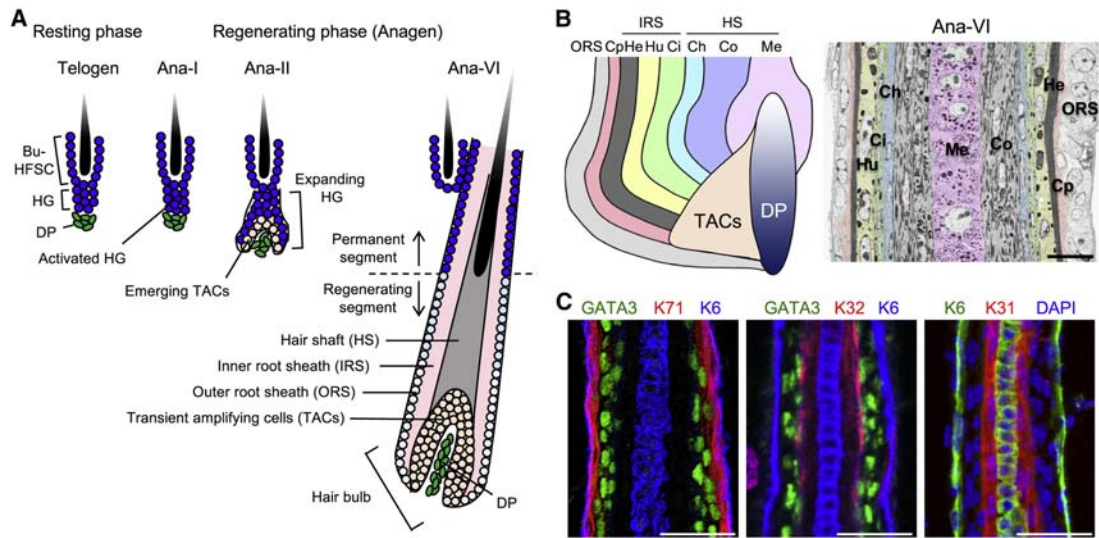


FIGURE 2.15

Modes of skin regeneration by skin stem cells (SCs). From Prodingner CM, Reichelt J, Bauer JW, Laimer M. Current and future perspectives of stem cell therapy in dermatology. *Ann Dermatol.* 2020;29:667–687.

**FIGURE 2.16**

The hair follicles regenerate throughout life and their structure is complex. From Yang H, Adam RC, Ge Y, Hua ZL, Fuchs E. Epithelial-mesenchymal micro-niches govern stem cell lineage choices. *Cell*. 2017;169:483–496.

becoming stem cells to regenerate the entire hair follicle structure. Interestingly, the complex interplay of the hair follicle stem cells does not contribute to the cells of the interfollicular skin layers in these studies. Thus, the dermal and hair follicle stem cells are different, although they are closely associated.

The replacement of skin following trauma, burns, or cutaneous ulcers has been performed for >100 years and is usually done by transplanting autologous skin from an uninvolved area. When surgery for skin replacement can be planned in advance, the skin can be encouraged to expand by placing a balloon under the skin and expanding the skin, causing the skSCs and skin TACs to proliferate, and full thickness skin can be harvested 3–4 weeks later for transplantation. Another approach is to harvest the donor skin area with a razor-like Dermatome that removes only a limited thickness “split skin,” and the skin can be “meshed” by short cuts that allow it to stretch over a larger wound area. The skSCs divide and migrate to fill-in the open mesh areas over a period of 3–4 weeks. Recent developments in understanding skSC have led to attempts to encourage in situ proliferation of wound-resident cells by treating a wound with growth factors.

2.16 $Lgr5^+$ stem cells of the intestine

The epithelium of the intestine constantly renews itself and is one of the most rapid tissues to turn over with the half-life of the intestinal epithelium being 4–5 days. The histology of the intestine is well studied, and it is understood that the cells of the microvilli are lost from the tip and replaced by cells originating at the base or “crypt.” The crypts have several identifiable cell types including the crypt base columnar (CBC) cells and the Paneth cells as well as the cells of the stalk originating at the base that

are known to divide frequently. As is often the case, the most proliferative cells are not the stem cells but the TACs of the stalk.

Through genomic sequencing, the LGR5 gene was first identified as an orphan receptor, a gene with the hallmarks of a cell membrane signaling receptor, but unknown ligand. The genes for the LGR class of receptors contain an exterior leucine-rich repeat domain coupled to an interior G-protein–coupled receptor sequence that triggers intracellular signaling for anabolic or catabolic activities. Using molecular biology gene engineering techniques to place the *lacZ* reporter gene into the last exon of the LGR5 gene and generating transgenic mice containing LGR5-*lacZ* expression, Hans Clever and colleagues at the Hubrecht Institute in Utrecht demonstrated that the LGR5-*lacZ* gene product was expressed in the columnar base cells but not the Paneth cells of the crypts (Fig. 2.17). With time, the *lacZ* positive (blue) cells of the crypt were seen to move up the villus toward the tip. Further, 4 h pulse labeling DNA with 5-bromodeoxyuridine labeled about one of the four CBC cells per crypt, while a 24 h labeling labeled ~4 CBCs/crypt, indicating the CBCs, were actively replicating with a replication period of ~24 h. In this figure, the first cells to show *lacZ*-labeled expression (stained blue in the figure) are the columnar base cells of the crypt (a) and not the neighboring Paneth cells or wall absorptive cells (b),

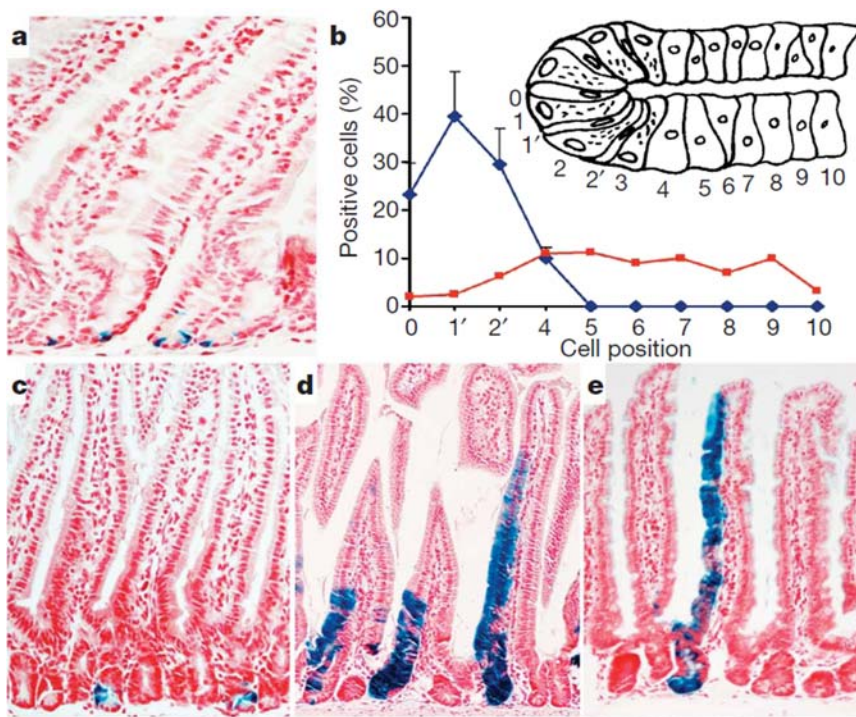


FIGURE 2.17

The intestinal microvilli have LGR5 expressing stem cells at the crypt base which become absorptive cells and migrate toward the tip where they are sloughed off through normal use. From Barker N, van Es J, Kuipers J. et al. Identification of stem cells in small intestine and colon by marker gene *Lgr5*. Nature. 2007;449:1003–1007. <https://doi.org/10.1038/nature06196>.

and the labeling is shown at 1 day (c), 5 days (d), and 60 days (e) as the cells migrate up the villi. Similar studies followed with other markers and conditional expression systems to corroborate the stem cell-like character of the crypt CBC cells, and Lgr5 as the best marker. Additional work also identified Lgr5⁺ cells in the crypts of the colon, although the colon has fewer crypts, and the rate of replication is much slower than the intestine. The expression of Lgr5 has been demonstrated in a variety of epithelial tissues including mammary glands and hair follicle and represent presumptive stem cells in these adult tissues.

Additional work demonstrated that the Lgr5⁺ cells were sensitive to radiation damage, but a second population of quiescent crypt stem cells was less sensitive. As shown in Fig. 2.18, the Lgr5⁺ labeled with green fluorescent protein (GFP) populates the cells of the crypts and lower villi (Fig. 2.18a); however, if the mice are exposed to 12 gray whole body irradiations, the villi Lgr5⁺ cells are not found (Fig. 2.18b) but the fully formed villi have prominent expression of the Bmi1 promoter-gene product (green cells in Fig. 2.18c/d) These radiation surviving cells could reform a complete villus and identified a second intestinal stem cell population that expresses Bmi1. While the Lgr5 population of intestinal

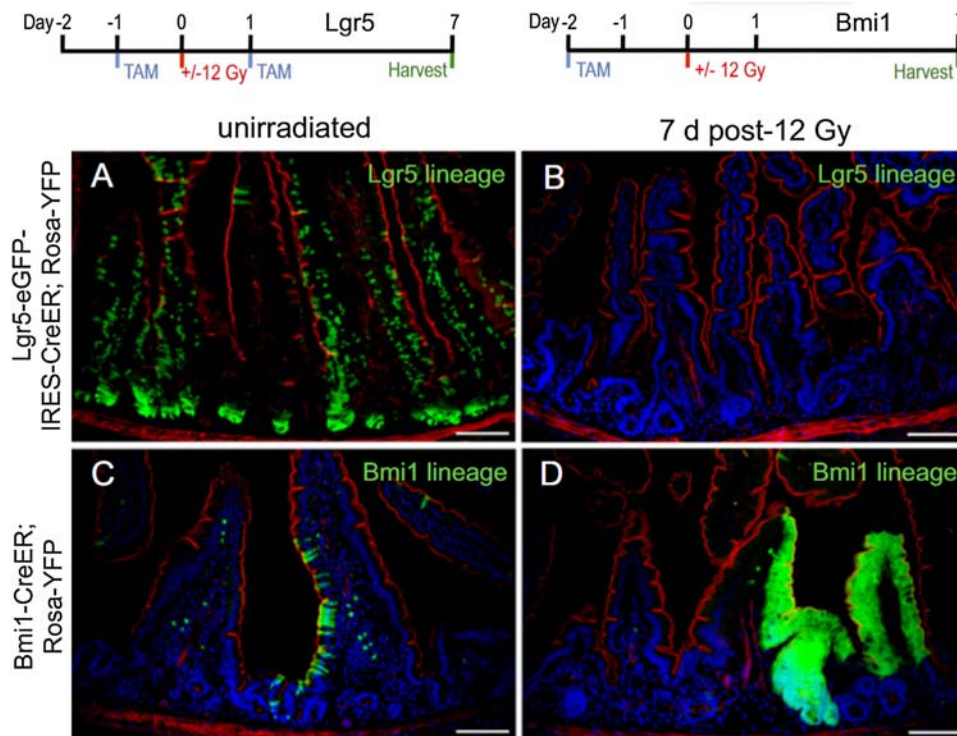


FIGURE 2.18

The intestinal crypt provides two populations of stem cells that are active in regenerating the villi. Normally, Lgr5 expressing cells are the most active (green cells in a), while Bmi1 cells provide only a small contribution (green cells in c), but if Lgr5 cells are ablated (b), the Bmi1 cells contribute strongly to villus maintenance (d). From Yan, Chia, Li, Ootani et al. *The intestinal stem cell markers Bmi1 and Lgr5 identify two functionally distinct populations*. Proc Nat Acad Sci. 2012;109:466–471.

stem cells appears to perform the routine replacement of intestinal epithelia cells, under conditions of injury this second population is activated. However, if the damage to the intestinal tissue is extensive or over a long period, both these cell populations may be inadequate, and scarring will be evident.

As a generalization, this concept of a routinely cycling stem cell population and a less prominent but important back-up population that becomes activated in response to injury is found in many tissues. That is, the body's normal repair and replacement mechanisms are not always enough for tissue repair at the time of massive cell loss due to injury or disease. Normal and pathology responses are both important in understanding the stem cells and tissue responses to every day needs and injury, as both may provide important avenues for intervention for the tissue engineer.

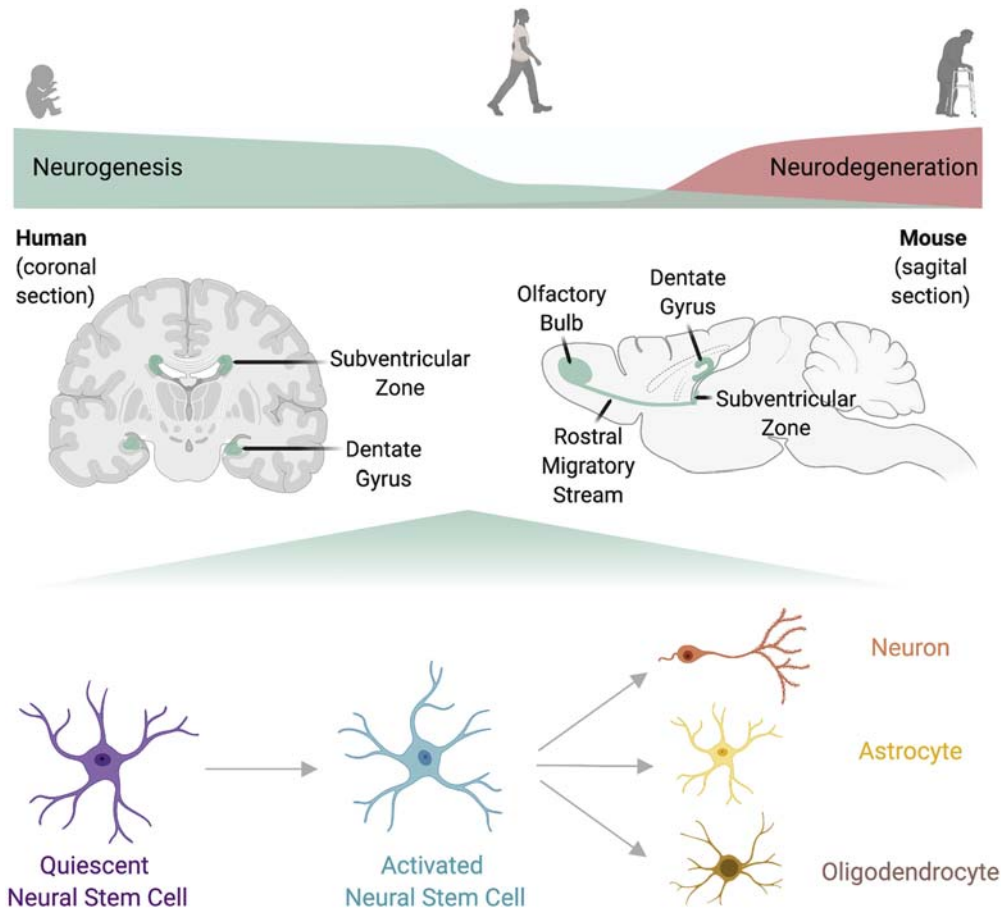
2.17 Central nervous system stem cells

The central nervous system contains stem cells. Several sites in the brain have been found to produce cells that can proliferate and differentiate to neuronal precursors or glial precursors (these form astrocytes and oligodendrocytes). The anatomical location that is the source of these stem cells is a small region in each hemisphere near the hippocampus known as the subventricular zone, a second site known as the dentate gyrus, and the olfactory bulb from which cells can migrate to the brain. From studies that label replicating cells, and then visualized their migration to sites of brain injury, it has been shown that some cell replacement, repair, and recovery of function are possible. It remains difficult to access these internal sites for delivery to optimize central nervous system regeneration. Over the last 20 years, significant research also has been performed on the spinal cord, the other half of the central nervous system. For spinal cord injury, there is not a recognized source of endogenous repairing stem cells as in the brain. Some regeneration following spinal cord injury has been demonstrated by placing exogenous progenitor/stem cells into the injury site, usually with the aid of a bioengineered matrix material. Like most injuries, lesions in the central nervous system seem to respond best to early treatment, before significant fibrosis has occurred (Fig. 2.19). Another approach to repair damaged neural tissue is to use pluripotent stem cells to generate neural precursor cells that can be used therapeutically in a similar manner to NSCs.

As of this writing in 2022, there were 75 clinical trials utilizing neural stem cells listed at clinicaltrials.gov including 24 that were listed as recruiting, enrolling, or active for conditions including Parkinson's disease, malignant glioma, ischemic stroke, multiple sclerosis, and spinal cord injury.

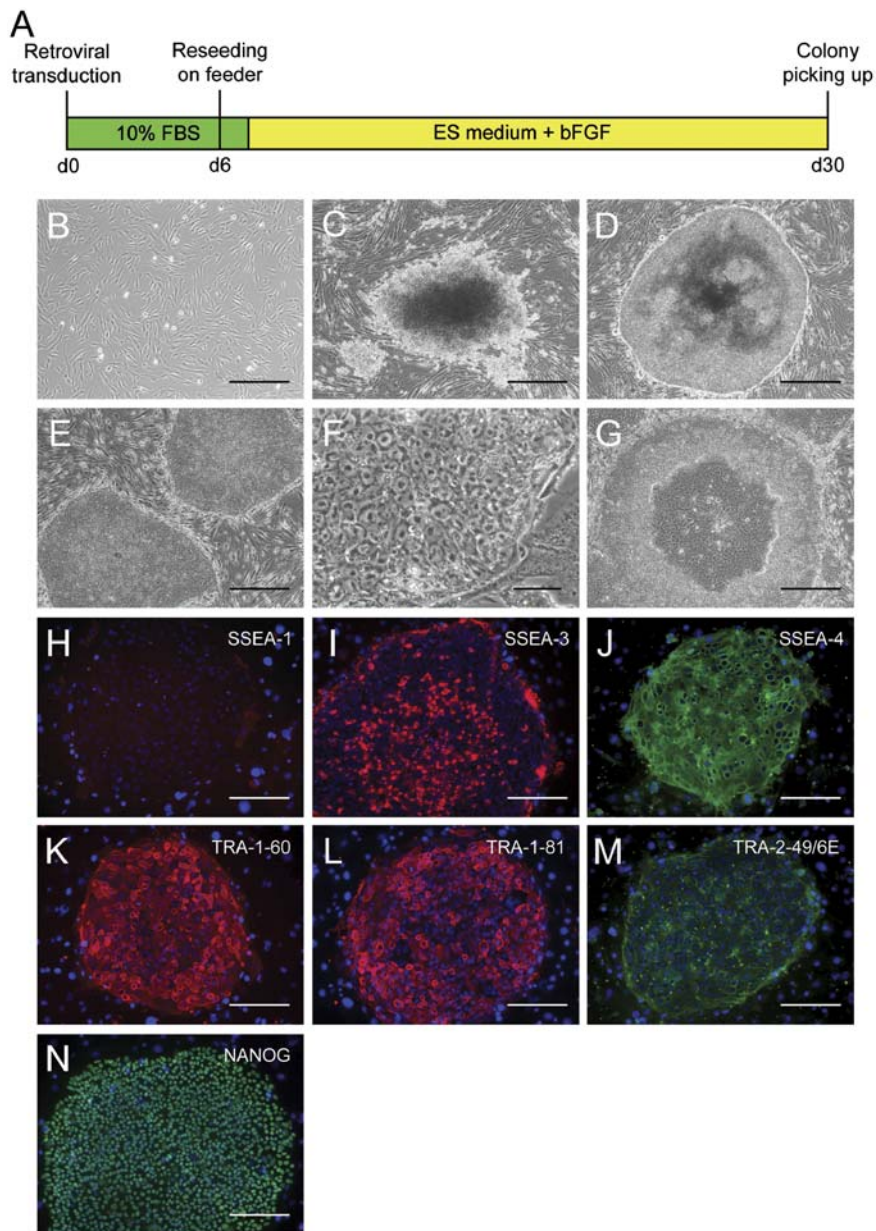
2.18 Induced pluripotent stem cells—iPS cells

Numerous molecular approaches and gene expression studies have identified genes associated with cell proliferation in normal and cancerous cells. These studies give clues to how stem cells control their proliferation and that it may be possible to activate adult cells to a stem cell-like phenotype by expressing the right gene products. Using this novel approach, Shinya Yamanaka, who received the Nobel Prize for Physiology or Medicine in 2012 for this work, demonstrated that by expressing genes known to promote the embryonic stem (ES) cell phenotype in somatic mouse and human fibroblast cells, they could convert them into ES-like clusters of cells. These cells are called iPSCs and they were originally generated by infecting a somatic cell with retroviruses carrying each of four transcription factors; Oct4 and Sox2,

**FIGURE 2.19**

Neural stem cells propagate during early development but are only found in a few brain sites in the brain during adulthood, and there is only a little central nervous system repair late in life. From Negredo et al. *Aging and rejuvenation of neural stem cells and their niches*. *Cell Stem Cell*. 2021;27:202–223.

which promote pluripotency in early embryos and ES cells, and the oncogenes KLF4 and c-Myc which were also known to contribute to the ES pluripotency and self-renewal. By retroviral infection with these four transcription factors in adult mouse and human fibroblasts, the differentiated fibroblasts were reprogrammed into a pluripotent state expressing the genes SSEA3, 4, Tra 1–60, Tra 1–81, and others (Fig. 2.20). However, the number of iPS cells generated remains low and about 160 colonies per 1 million target cells were produced. Later another research group demonstrated that adding two additional gene products NANOG and LIN28 could raise the number to 1600 per million target cells. Still a rather low number. The reasons for the low number of iPS cells generated from the best protocols remain under active investigation, but proliferation produces large numbers of these iPSCs.

**FIGURE 2.20**

Reprogramming of adult fibroblasts to induced pluripotent stem cells (iPS Cells). From Takahashi *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 131:1–12.

Since their conception, iPSCs have been generated by laboratories worldwide from a variety of species and using cells from patients with specific diseases to allow in depth study of the diseased state. To date, iPSCs have been generated by a variety of methods including adenoviruses and lentiviruses that express the genes regulating pluripotency, including Oct4, Sox2, Nanog, and Lin28 in addition to oncogenic factors such as c-Myc and Klf4. These studies show that pluripotent genes such as Oct4 and Sox2 and/or Nanog are critical for reprogramming, while c-Myc and Klf4 expression, though not required, significantly increases the reprogramming efficiency. Klf4 or c-Myc can also be replaced with small molecules that modify the epigenome such as histone deacetylase inhibitor, or by the SV40 large T protein which inhibits the cell cycle regulators p53 and retinoblastoma protein Rb. An issue is improving the low efficiency of converting somatic cells into iPSCs by these methods as laboratories report 0.001%–4% efficiencies rates. This inefficiency of iPS cell generation is also dependent on the cell source where less differentiated cell types (such as MSCs or fetal cells) generate iPS at higher efficiency than mature adult or aging cell types. The iPSC methods are also being applied to produce other types of stem cells with greater expansion potential such as the production of MSCs from iPSCs. With these iPSC-derived MSCs, it is reported they can be expanded successfully for more population doublings than the normal BM-MSCs.

In the laboratory, after transfection of the reprogramming factors into cells, stable iPSC lines can be detected in 2–4 weeks. At 2 weeks, individual colonies with tight compact morphology are carefully selected, dissociated, and used to generate stable cell lines. Selection of *bono fide* iPS colonies is critical as many colonies will never completely become pluripotent stem cells. For this purpose, human iPSC lines may be FACS sorted for cell surface marker expression such as TRA-1-60, TRA-1-81, and SSEA4. Although iPS cells display similar morphology and marker expression as ESCs, their genetic and epigenetic expression patterns are not identical and iPS cells retain some characteristic markers of their cell of origin and must be further characterized.

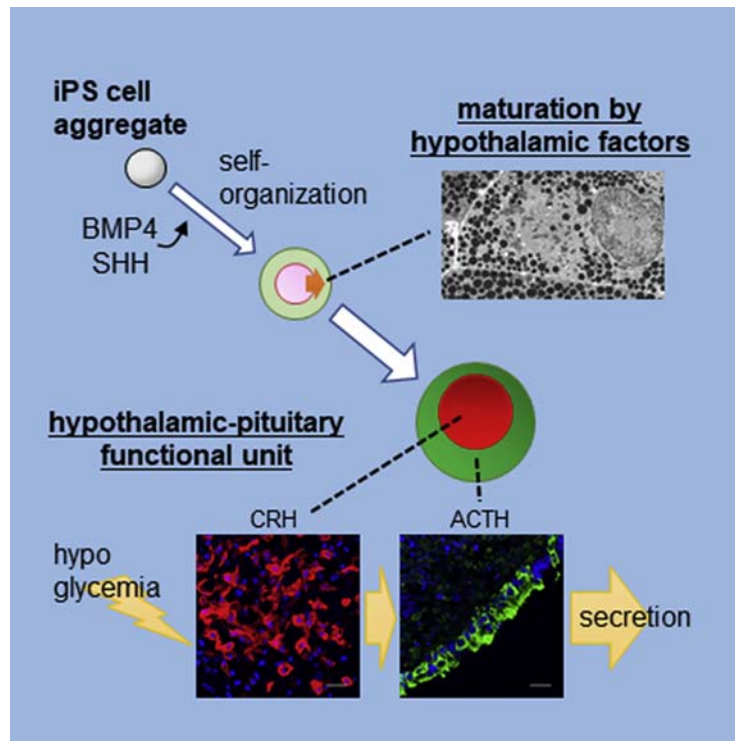
An important goal is to generate iPSCs within which the pluripotency genes can be turned off to allow their differentiation into functionally mature cell types, and to prevent the iPSCs from causing a teratoma or tumor formation after transplantation. To alleviate these concerns, great effort is ongoing to develop technologies that will allow the expression of pluripotent factors without the integration of foreign DNA into the host genome. These strategies include (1) transfections of the mRNA or proteins themselves (protein transduction) for the reprogramming factors; (2) small molecules (drugs) that facilitate expression of the reprogramming factors; and (3) the use of nonintegrating vectors (DNA footprint free technology) such as episomal plasmids that transiently express the reprogramming factors in the cytoplasm. These approaches may allow greater clinical use of iPSCs. The iPSCs are powerful tools for in vitro modeling, but their in vivo clinical use has been slow for safety concerns.

The multilineage differentiation potential of iPSCs may yet be a therapeutic blessing. The lack of a single lineage differentiation is cause for concern for therapeutic use but if accepted and thought of as a benefit, the iPSCs may yield new therapies for a large number of tissue types where there is promising animal data as shown in Fig. 2.21. Human iPSCs can form multilineage model systems of development and disease for a variety of somatic tissues. Differentiation protocols for the cell types comprising these somatic tissues have been developed and refined over the past 2 decades, initially through two-dimensional culture. In the past 5 years, the hiPSC field has experienced rapid developments in

	Tissue Type	Major Cell Types	2D Dual & Co-Culture	Microfluidic Chips	Tissue Engineering	Organoids/Assembloids	Animals & Chimeras
		Neuron Oligodendrocyte Microglia Astrocyte Endothelial cell	Brennan 2011 Israel 2012 Amin 2013 Wen 2014 Ishii 2017 Garcia 2019	Brown 2016 Wang 2017 Sances 2018 Vatine 2019 Park 2019	DeQuach 2011 Wang 2011 Zhang 2016 Appelt-Menzel 2018 Joung 2018 Cakir 2019	Lancaster 2013 Gabriel 2017 Birey 2017 Mansour 2018 Kanton 2019 Trujillo 2019 Harembak 2019	Nori 2011 Wang 2013 Chen 2016 Windrem 2017 Mansour 2018 Xu 2019
		Rod Cone Retinal ganglion Retinal epithelium Bipolar cell	Teotia 2017 Songstad 2017	Achberger 2019	Worthington 2017 Li 2017 Yang 2017 Calejo 2020	Zhong 2014 Foster 2017 Susaimanickam 2017 Hallam 2018 Achberger 2019	Carr 2009 Kanemura 2014 Kamao 2014 Zhao 2015
		Type I hair cell Type II hair cell Inner cochlear hair cell Outer cochlear hair cell Neuron	Gunewardene 2016 Chen 2018			Koehler 2017 Jeong 2018 Mattei 2019	Chen 2018 Takeda 2018
		Melanocyte Keratinocyte Merkel cell Langerhans cell Fibroblasts	Veraitch 2013	Abaci 2016	Itoh 2013 Petrova 2014 Gledhill 2015 Abaci 2016 Kim 2018	Kim 2018 Kim 2019	Itoh 2013 Yang 2014 Abaci 2016 Kim 2018 Liu 2019
		Lung epithelium Type I pneumocyte Type II pneumocyte Alveolar macrophage Endothelial cell	Gotoh 2014		Ghaedi 2013 Gilpin 2014 Wilkinson 2017 Ghaedi 2018	Gotoh 2014 Dye 2015 Wilkinson 2017 Miller 2019 Strikoudis 2019	Shafa 2018
		Cardiomyocyte Cardiac fibroblast Endothelial cell Smooth muscle Cardiac pacemakers	Josowitz 2016 Schweizer 2017 Yoshida 2018 Zhang 2019	Wang 2014 Ellis 2017 Zhang 2017 Kalmikov 2019	Mathur 2015 Ong 2017 Serpooshan 2017 Shadrin 2017 Tiburcy 2017 Ani 2018 Ronaldson-Bouchard 2019	Voges 2017 Mills 2017 Hoang 2018 Mills 2019	Ye 2014 Chong 2014 Funakoshi 2016 Kadota 2017 Liu 2018 Bargehr 2019
		Lymphocyte Monocyte Granulocyte Erythrocytes Endothelial cell	Choi 2009 Dias 2011 Adams 2013 Yang 2014 Patsch 2015	Zanotelli 2016 Ribas 2017	Samuel 2013 Gui 2016 Atchison 2017 Skylar-Scott 2019 Atchison 2020	Wimmer 2019 Montel-Hagen 2019 Motazedian 2020	Orlova 2014 Tan 2018 Espinoza 2018
		Osteoblast Osteoclast Myoblast Tenocyte Chondrocyte	Qu 2013 Demestre 2015		de Peppo 2013 Wang 2014 Kang 2014 Jeon 2016		de Peppo 2013 Phillips 2014 Jeon 2016 Sheyn 2016
		Mucous cell Parietal cell Chief cell Gastrin cell Neuroendocrine cell		Lee 2018		McCracken 2014 McCracken 2017 Trisno 2018 Zhang 2018 Broda 2019	
		Alpha cell Beta cell Delta cell Gamma cell Stellate cell	Trott 2017	Tao 2019	Wan 2017 Kuo 2017 Enderami 2018 Abazari 2018	Pagliuca 2014 Raikwar 2015 Huang 2015 Hofweiler 2017 Tao 2019 Alvarez-Dominguez 2020	Kroon 2008 Kobayashi 2010 Jeon 2012 Ma 2018 Haller 2019
		Hepatocyte Stellate cell Kupffer cell Oval cell Endothelial cell	Ware 2015 Berger 2015 Freyer 2017	Schepers 2016 Wang 2018 Ramme 2019	Ware 2015 Faulkner-Jones 2015 Ma 2016	Takebe 2013 Guan 2017 Takebe 2017 Koike 2019	Liu 2011 Takebe 2013 Nagamoto 2016 Pettinato 2019
		Podocyte Endothelial cell Parietal cell Brush border cell Mesangial cell	Xia 2013 Taguchi 2014 Toyohara 2015 Tajiri 2018	Musah 2017 Ramme 2019	Batchelder 2015 Fischer 2017 Benedetti 2018	Morizane 2015 Takasato 2015 Forbes 2018 Czerniecki 2018 Low 2019	Toyohara 2015 van den Berg 2018 Subramanian 2019 Low 2019
		Enterocyte Goblet cell Enteroendocrine cell Paneth cell Tuft cell	Zhang 2018 Takayama 2019	Workman 2018 Sidar 2019 Ramme 2019	Finkbeiner 2015 Kitano 2017 Workman 2017	Spence 2011 Workman 2017 Crespo 2017 Munera 2017 Takahashi 2018 Mithai 2020	Watson 2014 Jung 2018

FIGURE 2.21

Human iPSCs can form multilineage model systems of development and disease for a variety of somatic tissues. *From Sharma A, Sances S, Workman MJ, Svedsen CN. Multi-lineage human iPSC-derived platforms for disease modeling and drug discovery. Cell Stem Cell. 2020; 26:309–329.*

**FIGURE 2.22**

iPS cell aggregates differentiated to release hormones such as CRH and ACTH. *From Kasai et al. Hypothalamic contribution to pituitary functions is recapitulated in vitro using 3D-cultured human iPS cells. Cell Rep. 2020;30:18–24.*

organoid, tissue engineering, and microfluidic chip models. Key studies utilizing hiPSC-derived cells in a multilineage context are listed according to tissue type and culture platform. As a further example of this concept, researchers have created an in vitro hypothalamus–pituitary “gland” capable of secreting both corticotropin-releasing hormone (CRH) and adrenocorticotrophic hormone (ACTH) as shown in Fig. 2.22. It is difficult to isolate pure iPSCs of one lineage, and aggregates will differentiate to several lineages. Here, iPS cell aggregates were treated with factors to promote hypothalamus differentiation and then pituitary promoting factors such that the pituitary-like aggregate centers can be prompted by hypoglycemic conditions to produce CRH, which then induces release of ACTH from the outer cells.

2.18.1 Deextinction of the northern white rhino by iPS cells

The northern white rhino is all but extinct; the last, two and one-half ton male named Sudan died in 2018 at the age of 45. The habitat range of this animal was reduced by national conflict, clearing land for farming and poaching for its prized horn. No animals exist in the wild. The remaining four females, which have horns too, are all under constant protection in wildlife parks (Fig. 2.23). Researchers are creating iPS cells from the rhino’s cryopreserved skin cells to create an embryo. Next, the newly created embryo will be placed in a surrogate southern white rhino mother which is not endangered. There are challenges, and the gestation is a long 16 months. A new implantation catheter design is needed for this work in these large animals as well. Several animals would need to be created before a breeding

**FIGURE 2.23**

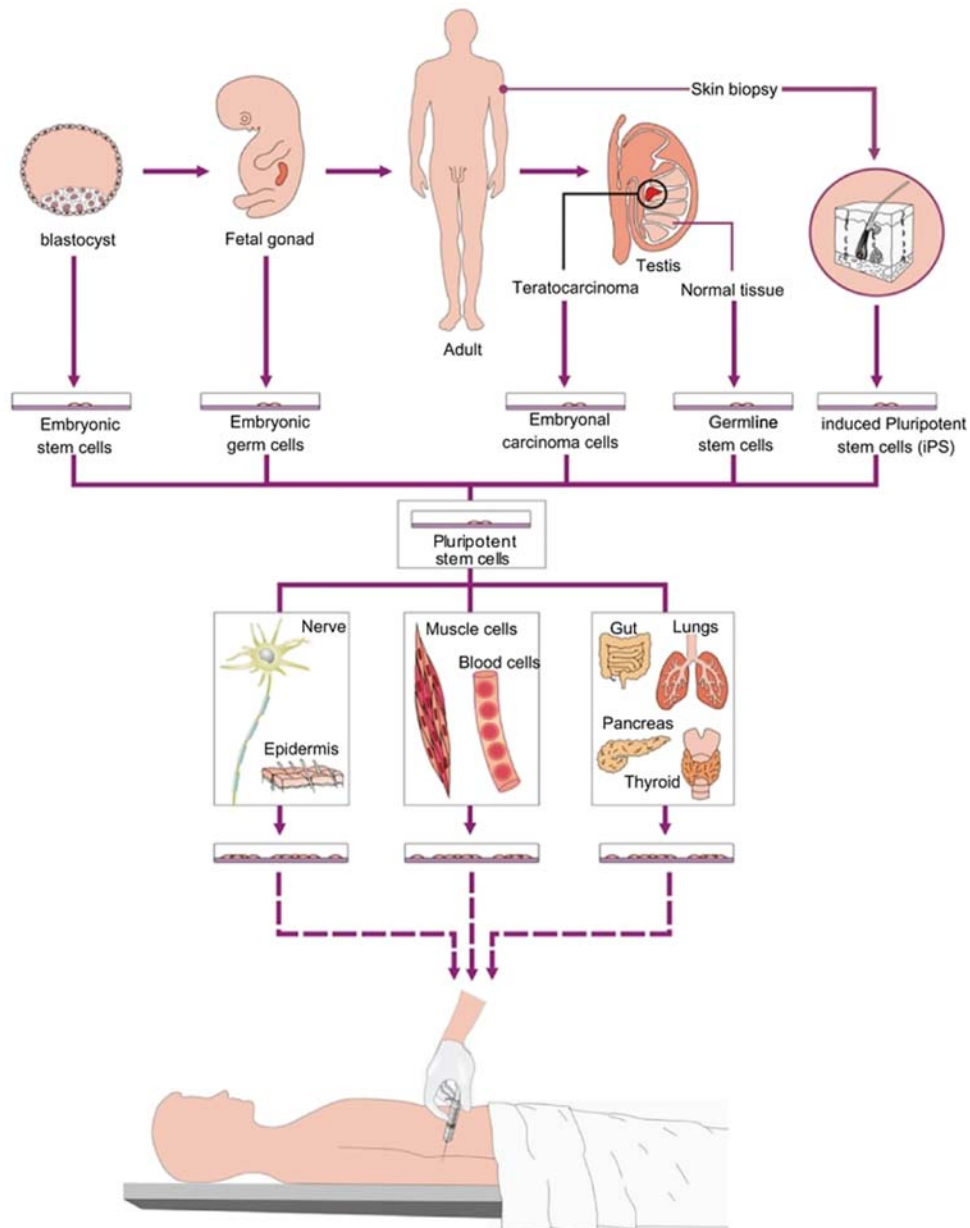
This is the northern white rhino, and the last male died in 2018. Now researchers are planning to use his frozen skin cells to create iPS cells that may then be used to create a new embryo that can develop after implantation in a surrogate mother. From *Vertigo_Warrior. The end of an era*; 2021. *Twitter.com* <https://twitter.com/VertigoWarrior/status/1399988901750599681>.

program could proceed. This noble endeavor could take 15–20 years, but most agree it is worth the effort to save these magnificent animals that have been pushed to extinction by human activities, a win for biologists, conservationists, and all animal lovers.

2.19 Natural pluripotent and embryonic stem cells

Prior to the development of iPS cells, pluripotent stem cells were isolated from embryos or from germ cell progenitors of sperm or egg. These *in vitro* propagated stem cells include ES cells, embryonal carcinoma (EC) cells, and embryonic germ (EG) cells. These cells have been derived from a multitude of species including rodents and humans. ESCs can be isolated from the inner cell mass of a normal early embryo or from trophoblast cells or unfertilized, parthenogenetically activated eggs. Cells from these sources are transferred to a culture dish with the proper media and growth factors (Fig. 2.24). Human pluripotent stem cells include ESCs cultured from cells of the inner cell mass of normal or parthenogenetic blastocysts, embryonic germ cells generated from primordial germ (PG) cells in late embryonic development, embryonal carcinoma cells isolated from adult teratocarcinomas, germline stem cells derived from spermatogonia, and iPSCs generated by reprogramming differentiated adult cells. Pluripotent stem cells exhibit the potential to produce all cell types of the body. Thus, directed differentiation of these cells holds promise for treating a wide variety of diseases and injuries. The ES cells established many of the paradigms for stem cells and are still teaching us new lessons about early development, stem cell communication, regulation of early gene expression regulation, and aging.

Pluripotent stem cells can also be derived from germ cells and their progenitors. Embryonal carcinoma (EC) cells were the first pluripotent stem cells to be identified in the 1960s, originally from the mouse and later from human tissue. EC cells are pluripotent cells derived from adult testicular teratomas (benign tumors) or teratocarcinomas (malignant tumors) originating from undifferentiated PG cells. PG cells are the progenitors of the germ cell lineage and are unipotent. However, under the appropriate environmental cues, PG cells can revert or dedifferentiate into pluripotent stem cells. This ability is also

**FIGURE 2.24**

Sources of pluripotent stem cells for therapeutic use. From Swelstad, Kerr. *Stem cells and cloning: advances and applications*. 2010;3: 13–27.

demonstrated in vitro from PG cells isolated from late embryonic or early fetal period which can be used to generate pluripotent stem cells called embryonic germ (EG) cells. ES, EC, and EG cells share similar features with iPSCs that have largely replaced them. EC cells are cancer stem cells and are karyotypically unstable rendering their use in development studies confounded with genetic abnormalities and not appropriate for any potential use in stem-cell based therapies or tissue engineering. While not derived from an embryonic source, pluripotent stem cells have also been derived from a few laboratories from spermatogonia stem cells called germ-line stem cells from adult testes as well.

2.19.1 Differentiation of pluripotent stem cells

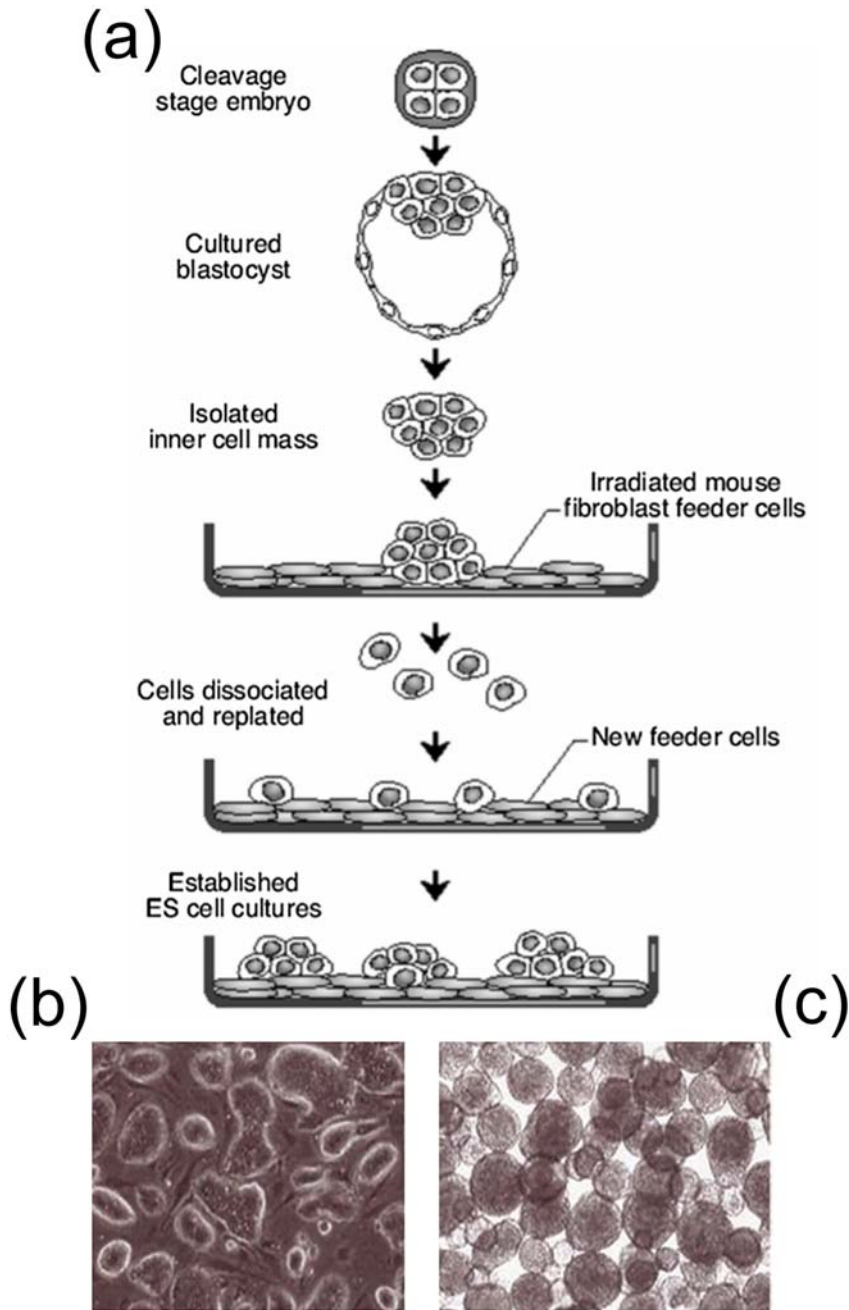
Human pluripotent stem cells might be an ideal cell source for tissue engineering and regenerative medicine, because of their indefinite proliferation capacity and pluripotency (Fig. 2.25). Pluripotent stem cells are often mentioned as a promise for the cure of macular degeneration, Parkinson's disease, diabetes, and cardiovascular diseases. This inevitably means that these cells must be differentiated into respectively retinal epithelial cells, neurons, insulin-producing cells, and cardiomyocytes. Many articles are published in which the in vitro and in vivo differentiation of human pluripotent stem cells is described. Useful cell types such as neurons, cardiomyocytes, hepatocytes, pancreatic beta cells, endothelial cells, blood cells and chondrocytes have all been successfully derived in the laboratory. The in vitro differentiation of human ES cells on polymeric scaffolds into 3D structures with characteristics of developing neural tissues, cartilage, liver, or blood vessels has been published.

2.19.2 Application of pluripotent stem cells

It is obvious that mouse pluripotent stem cells will never find clinical applications. However, the knowledge of mouse pluripotent stem cells was used for the isolation, growth, and differentiation, and thus, development of future applications with human pluripotent stem cells. Furthermore, mouse ESCs and iPSCs are used as model systems to study early embryonic development and differentiation.

Another valuable feature of mouse pluripotent stem cells is the ability to create genetically modified mice. When genes are modified or new genes introduced into a pluripotent stem cell, they make a chimeric animal, and it is possible to breed a line of genetically changed mice. In a **knock-out mouse**, the gene product is not produced, and the biological function of the gene can be inferred. Similarly, a knock-in mouse can be genetically engineered to over produce a gene of interest, or a conditional knock-in mutant can be created in which the gene product can be turned on or off. When a mutation is introduced that is known to be the cause of a human genetic disease, the mice may serve as a model for this human disease. Much of the knowledge of stem cell self-renewal is also based on the use of these knock-out, knock-in, conditional knock-in and gene overexpression models.

Human ESCs and iPSCs can be used as a model system to study human embryonic development, differentiation, and disease (Fig. 2.26). While mouse models provide critical information regarding our current understanding of human biology, the ability to study human cells and their development is imperative to fill gaps in our understanding specific to human biology. In addition, iPSCs have also been generated from patients with a variety of diseases including Parkinson's, multiple sclerosis, sickle-cell anemia and diabetes. Thus, iPS lines will make it possible to study the development of these diseases in vitro. However, to use human ESCs or iPSCs in therapeutic applications, optimization of

**FIGURE 2.25**

Embryonic stem cell derivation method and cultivation. (a) Diagram of derivation of embryonic stem cells. (b) Colonies growing on a dish or (c) free-floating colonies.

their expansion and differentiation into function cell types is required. Most current methods for deriving these lines and maintaining them in culture are time consuming, labor intensive, and the chemically defined media is expensive.

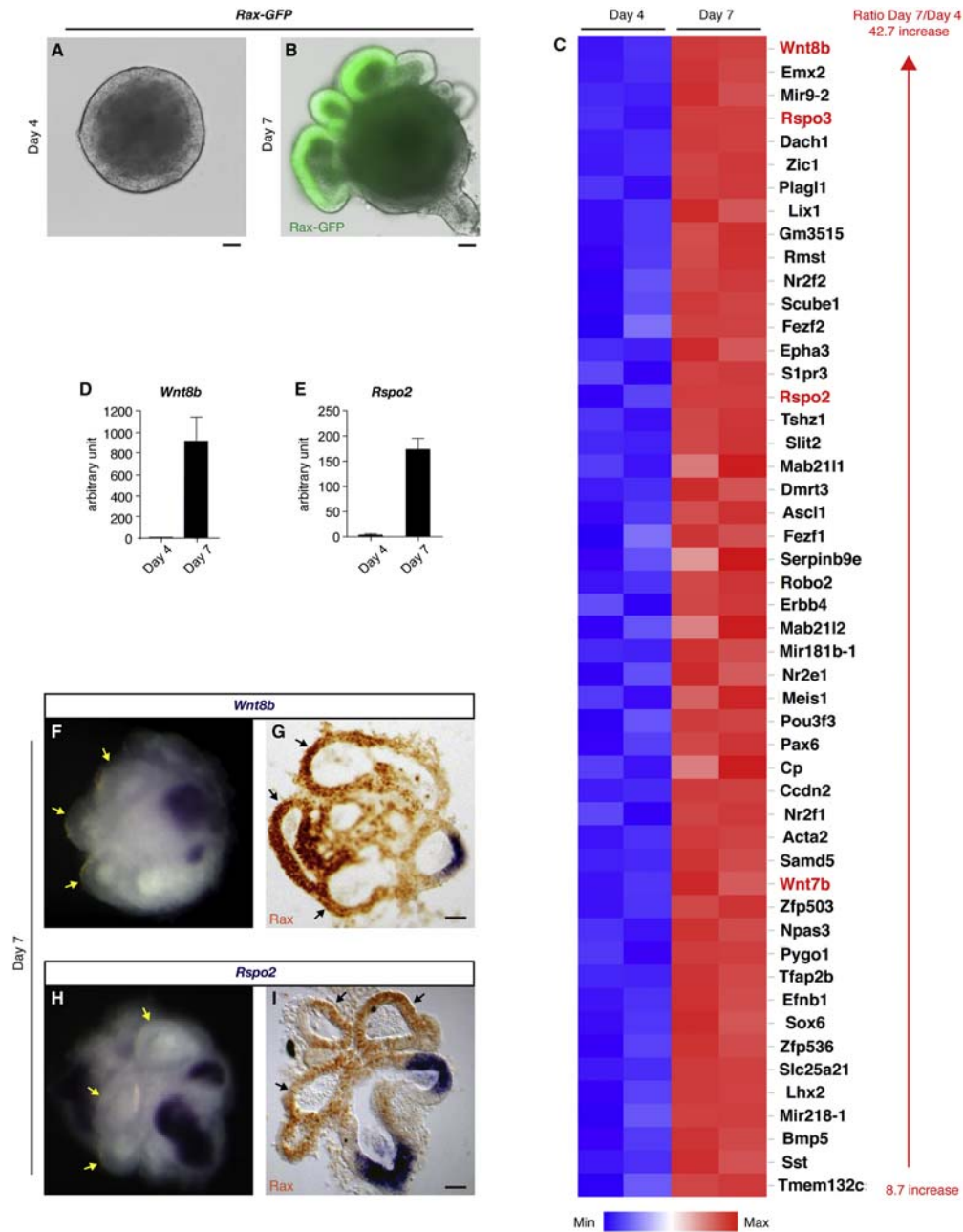
A major bottleneck for using these cells is the ability to derive and grow them without animal products. After the discovery of prion diseases, such as Creutzfeldt-Jakob (Bovine Spongiform Encephalopathy or mad cow disease), the risk of infection by nonhuman pathogens is well recognized. It is not just the animal sera used in culture that contaminate the human ES cell cultures. The extracellular matrix proteins that may replace the feeder cells are also usually from animal sources. Thus, some companies have now developed methods that mitigate these concerns by developing products to grow cells without the need for feeder layers or human or animal products.

2.20 Organoids, exosomes, and extracts from stem cells

Organoids are self-organizing multicellular aggregates or "mini-organs" that have similarities to complete organs. While it is not yet possible to build whole organs, it is possible to grow small multicellular pieces of organs that perform organ functions. It is also possible to grow stem cells and cause them to differentiate to functional organ parenchymal cells that produce cytokines and enzymes and perform metabolic functions and undergo morphological differentiation. These two methods can create organoids that are useful to understand organogenesis, organ function, and to study as an organ surrogate for drug testing. In [Fig. 2.26](#), mouse ESCs containing the GFP under control of the Rax retinoid homeobox promoter are cultured as organoids and induced to differentiate to a neuroretina lineage and analyzed at 4 and 7 days of differentiation. Panel A and B show the morphological changes and the increase in GFP expression. Panel C demonstrates the increased expression (blue to red) of the top 50 genes showing 8–42-fold increase, including several Wnt and R-spondin genes. In panels F and H, whole mount organoids show regions of high expression of Wnt8b and Rspo2, but cryosections (G and I) through organoids and hybridization to these genes show that the Rax antibody immunostaining (brown) is extensive but outside of the Rax + optic vesicle-like loops.

As another example, patient-derived organoids have been examined as surrogates for predicting treatment outcomes for advanced cancer patients. Organs-on-a-chip is another concept that seeks to control the biological environment and measure input/output parameters from organoids of differentiated stem cells. Organoids are usually only a few millimeters in diameter and can be cultured for days to weeks for short-term and long-term studies. 3D bioprinting has made it possible to plan and execute sophisticated studies on organoids. Current studies implant organoids in animal models to provide some functional benefit although this is usually limited. Nevertheless, this is a line of investigation toward future organ development for the tissue engineer.

Exosomes are subcellular membrane bound extracellular vesicles of ~50–150 nm that bud off from the cell membrane and carry a variety of cell contents as cargo including proteins, nucleic acids, and metabolites. They can survive in the circulation for 1–2 days and travel long distances in the body. The medicinal effects of exosomes are being explored and may be similar to the cells from which they are derived, and were it to be a successful therapy, this would avoid some problems of cellular therapies. For example, as shown in [Fig. 2.27](#), mesenchymal stem cells produce exosomes that contain

**FIGURE 2.26**

Organoid culture and ES cell differentiation to optic cells. Organoid culture of ESCs transduced with GFP under control of the retinal homeobox gene *Rax* promoter sequence analyzed at 4 and 7 days of optic cell differentiation. From Takata *et al.* Cell Rep. 2017;21:1534–1549.

micro-RNAs and proteins that influence inflammation, angiogenesis, fibrosis, and immune responses locally and at a distance. These exosomes are under evaluation as therapeutics thereby avoiding the transfer of whole cells with their genetic material. However, the production of exosomes in large amounts has many of the complexities that accompany commercial cell production and the release criteria of such products for clinical use are not yet established.

Extracts or conditioned medium from cultured stem cells are also being examined for their therapeutic potential. While thoughts of stem cell therapy evoke a cell for cell replacement of damaged cells in tissues and organs, stem cells also produce relatively complex mixtures of growth factors and cytokines that function in tissue repair and regeneration. These extracts or conditioned medium can be biochemically characterized for reproducibility, stability, and potency without many issues that accompany

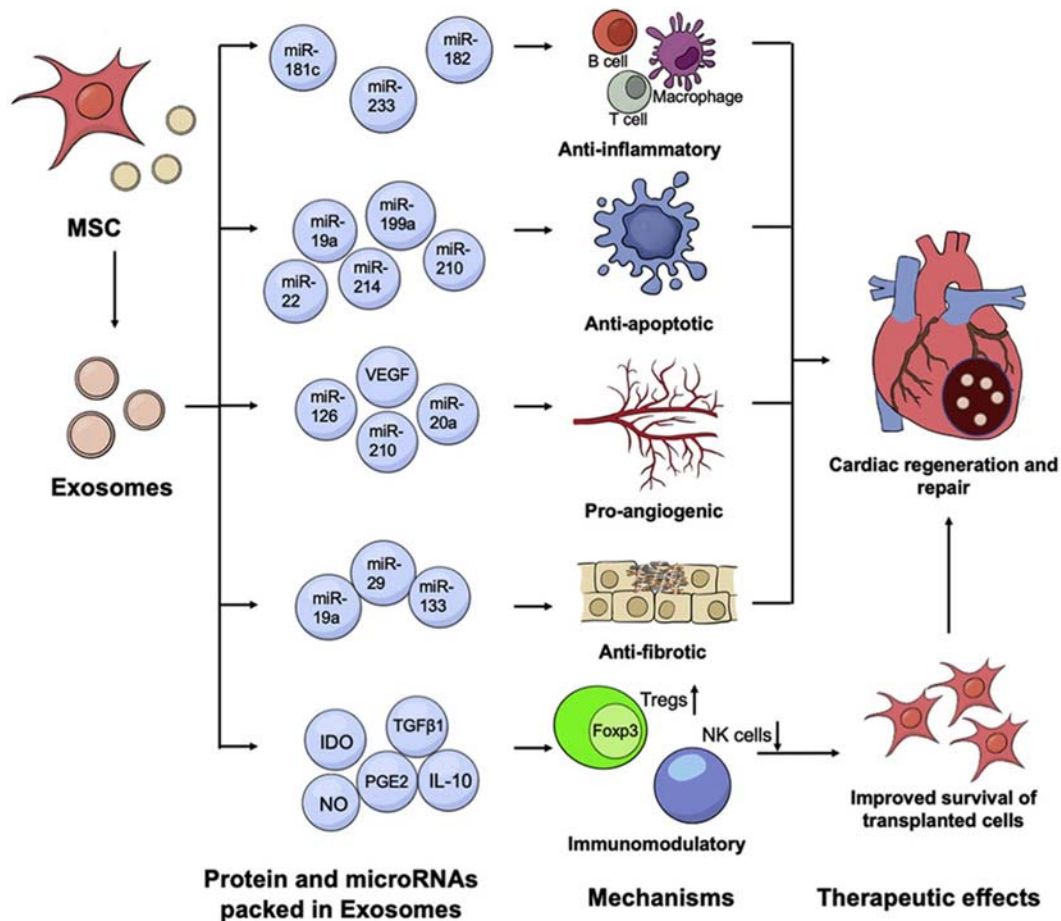


FIGURE 2.27

Exosomes produced by MSCs and other stem cells can carry proteins, micro-RNAs, and metabolites to nearby cells or over great distances to effect changes in other tissues. *From Sun et al. Mesenchymal stromal cell-derived exosomes in cardiac regeneration and repair. Stem Cell Rep 2021;6:1662–1673.*

cellular therapeutics. The regulatory pathway for clinical use of stem cell extracts or conditioned medium also requires rigorous evaluation and is subject to federal drug agency approval.

2.21 Stem cell mechanobiology: stretch and strain

Stem cells, both in bone marrow and within other organs, have surface receptors connected to actin and tubulin-based cytoskeletons and can sense changes in the extracellular matrix. These connections allow cell sensing of two-dimensional or three-dimensional stretch and strain occurring nearby, and this mechanosensing can alter mitosis, migration, and differentiation.

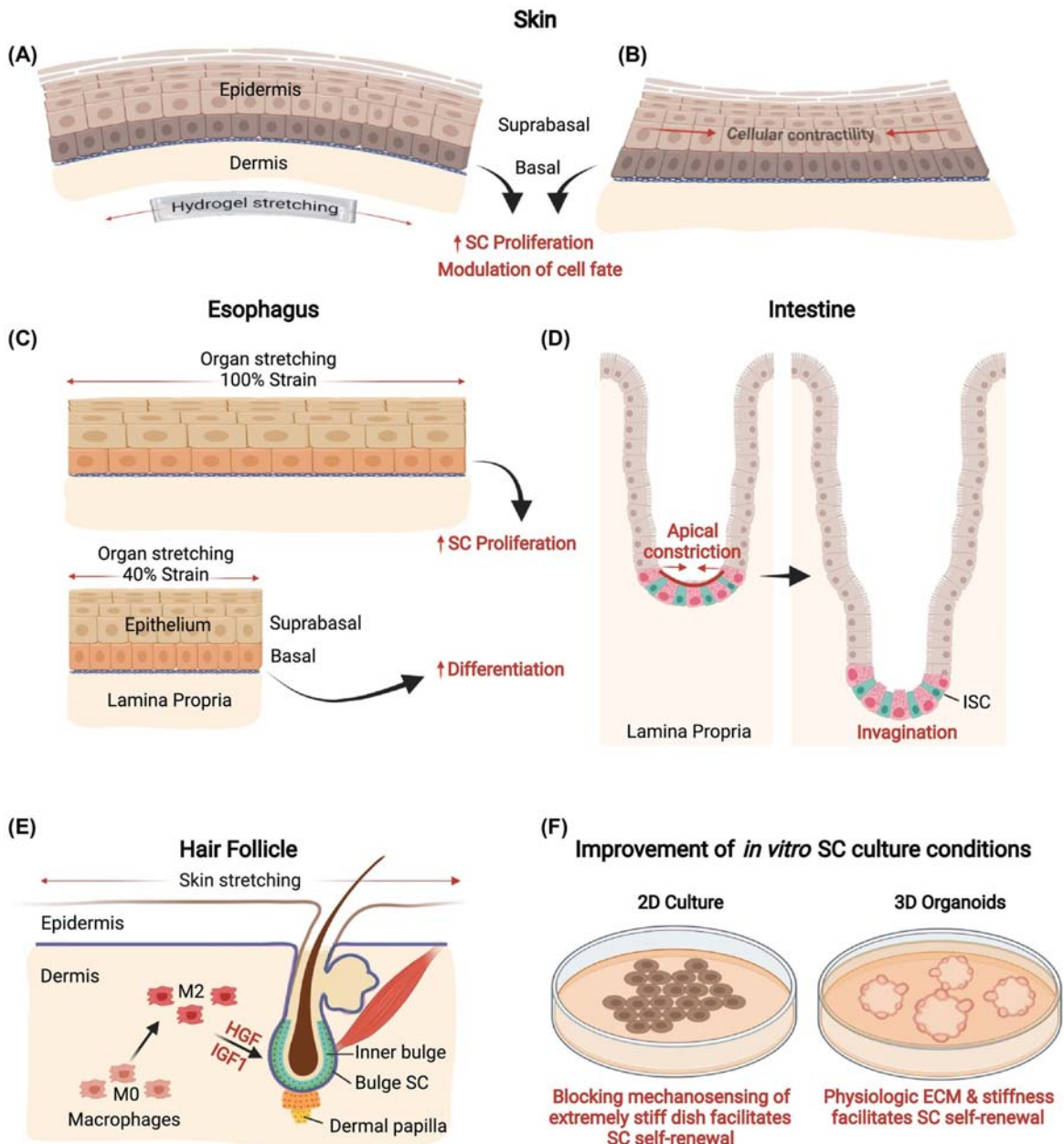
For example, prior to skin grafting, extra skin production can be stimulated by injecting a hydrogel under the skin to stretch it and induce epithelial proliferation. Similar examples are found in the esophagus, intestine, and hair follicle (Fig. 2.28). The sensing and intracellular signaling underlying these responses are partially known through biochemical manipulations and gene knock-out studies and involve cytoplasmic and nuclear events orchestrated by genes Rac, Rho, YAP/TAZ, and others. These types of responses are known for mesenchymal stem cells where cyclical stretching aids tendon-like differentiation and maturation, and for epithelial stem cells of skin, hair follicle, esophagus, and intestine. More recently, in vitro culture systems using organoids are being used to simplify and sort out these complex responses to mechanical manipulation.

2.22 Future perspective

An incredible amount of new research is now being performed on stem cells, including adult stem cells, induced stem cells, and ESCs. Many new studies in tissue engineering involve new materials in combination with stem cells but much remains to be discovered. The next decade or two will see greater understanding and implementation of stem cells for tissue engineering. Clinical trials that test effects of stem cell treatment are underway in most medical areas. The US government site that tracks most trials throughout the world (www.clinicaltrials.gov) lists almost 9000 clinical trials utilizing stem cells of one type or another, but not all cell types are equally represented—searching HSCs find almost 4400 trials, while the much newer MSCs find almost 1400 trials. Some are ongoing trials, while others are completed or not recruiting. Nevertheless, the enthusiasm to utilize stem cells as the latest tool to address unmet medical needs is clear and seems to be growing.

2.23 The dark side: cancer stem cells

We have discussed the excitement and promise of stem cells for tissue healing, but as with any powerful technology there are potential problems to understand. Stem cells are not cancer cells but share certain growth properties with cancer cells. Cancer cells proliferate, some rapidly and some slowly, and most are partially differentiated. There are some, such as leukemic cells, that replace normal cells and become the dominate cell in the tissue in which they are growing. Many anticancer treatments are designed to interfere with proliferation, such as nucleoside analogs that mimic the normal A, T, G, C-nucleotides and once incorporated into the replicating DNA cause lethal chain termination. Thus, certain cancers respond very well to certain drugs. However, much like the stem cell in its niche that divides only infrequently, a cancer stem cell may reside in an unknown niche location and produce daughter cells infrequently. It is the rapidly growing daughter cells that may be diagnosed and treated but it is thought to



Trends in Cell Biology

FIGURE 2.28

Stem cells are continuously monitoring their environment and do respond to mechanical stimulation as well as cytokines and growth factors. The cells' actin and tubulin cytoskeletons are connected to the extracellular matrix as well as the cell nucleus and stimulation often results in proliferation. From Bhattacharya S, Wolfenson H, Shivdasani R, Shalom-Feuerstein R. Stem cell responses to stretch and strain. Trends Cell Biol 2022;32:4–7.

be the underlying cancer stem cell that is at the source of the problem. Cancer cells that form solid tumors lack contact inhibition and continue to grow, although angiogenesis may not keep pace with tumor growth and the tumor may become self-limiting unless new blood vessels form.

Using a genetic Cre/LoxP recombinase cassette system, it is possible to label individual cells with one of four colors (confetti labeling or Brainbow). That is, there are four distinct LoxP sequences, and when the Cre recombinase causes excision, the intervening DNA sequence is removed, leaving only the one color adjacent to the promoter to be expressed. Labeling mouse mammary cancer cells by this technique and visualizing the same region of cells in the living mouse tissue by intravital microscopy over 2 weeks, it is possible to watch cancer cell dynamics (Fig. 2.29). The collected data in Fig. 2.29 shows some clonal

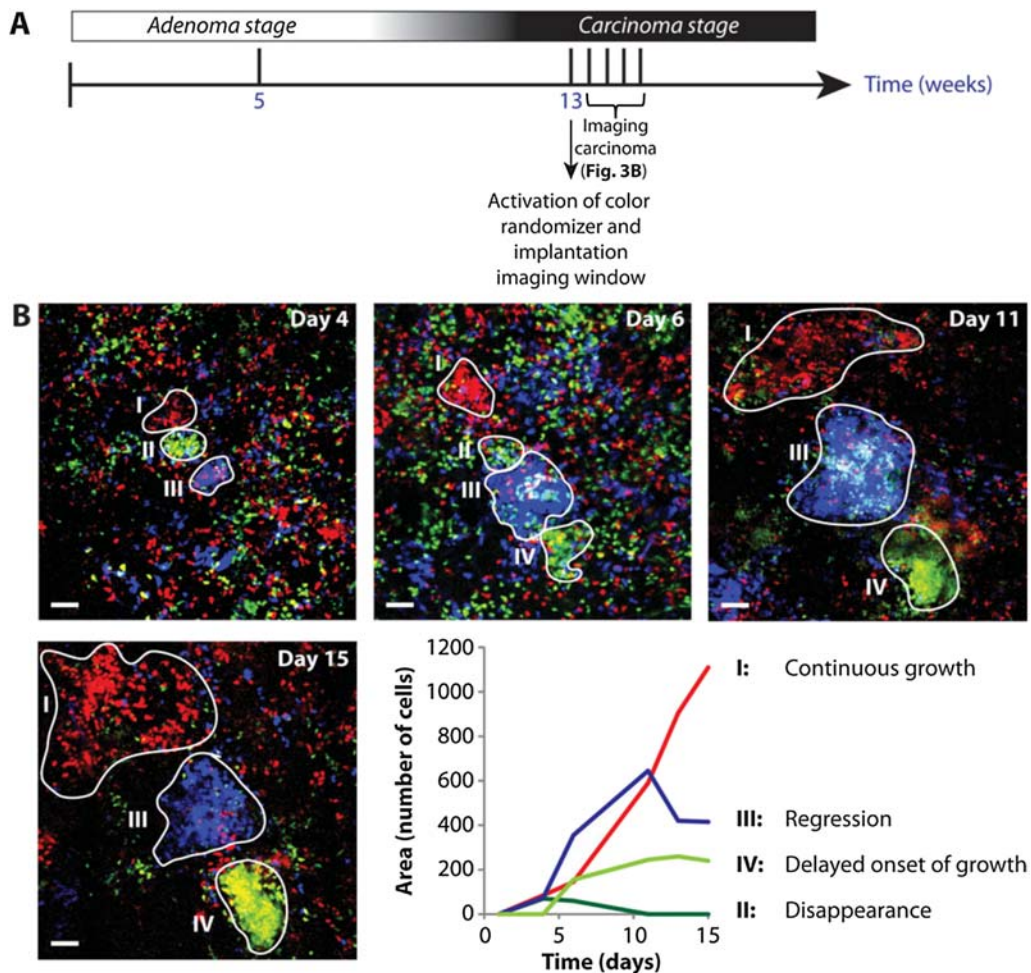


FIGURE 2.29

Intravital imaging of cancer stem cell dynamics. The mice are genetically engineered with a confetti cassette that results in expression of one color per tumor cell following brief treatment with tamoxifen. Individual colonies then grow from those tumor cells and can be imaged by intravital microscopy. Over 2 weeks, some cancer cell clones continued to grow (red), grew then regressed (blue), were delayed in their growth (yellow) or never grew (green). From Zomer et al. *Intravital imaging of cancer stem cell plasticity in mammary tumors*. *Stem Cells*. 2013;31(3):602–606. <https://doi.org/10.1002/stem.1296>. PMID: 23225641; PMCID: PMC3744756.

cancer stem cells expand in an uncontrolled manner (cells labeled in red), while others proliferate with the clonal colony size enlargement and then regress (blue), or others that demonstrate delayed proliferation onset (yellow), or other clones that only show a regressive phenotype (green). The data also indicates that a clone may remain small for a long period but then be triggered to continue growing at a later time, as is common in cancer relapse. Normally, stem cells and their progeny exhibit controlled growth, and are commonly produced and transplanted without signs or evidence of genetic changes in vitro or in vivo. Animal studies prior to any human trials also guard against developing deleterious cell transplantation effects at the clinical trial stage. Steps to watch for any deleterious cells arising in a stem cell population or final product are commonly part of stem cell therapy development.

Summary

- Stem cells are defined by the properties of self-renewal, proliferation, and differentiation.
- Continually renewing tissues such as blood, skin, hair, and intestinal epithelia have abundant stem cells, and these tissues usually repair quickly. Complex large structural tissues such as heart and brain are slow to repair or regenerate and have few stem cells, or, if present they may be quiescent, and difficult to activate for repair purposes.
- Apoptosis, autophagy, and necroptosis are processes of programmed cell death from within, while necrosis is cell damage caused by external factors such as trauma or cell membrane rupture.
- Stem cells are found in stem cell niches and interactions with neighboring cells may maintain them in a primitive state.
- Stem cells can be characterized by their proliferation and differentiation. They express certain genes, RNA, and proteins that are used to identify potential stem cells, but appropriate differentiation must also be evident.
- Bone marrow contains several useful adult stem cells: HSCs and mesenchymal stem/stromal cells (MSCs).
- HSCs may be the best studied adult stem cells and are present in bone marrow transplantation. However, they are difficult to grow in culture while maintaining their ability to differentiate. MSCs are 10-fold rarer than HSCs in bone marrow, but they are relatively easy to propagate in culture and provide useful precursor cells for many connective tissues including bone and cartilage.
- The stem cells of skin and the intestinal epithelia are extensively studied and serve as a model system for tissue repair and regeneration both from a scientific and a clinical perspective. The *Lgr5/Wnt/β-catenin* pathway is important for controlling gene expression in these stem cell systems.
- Adult stem cells are widely used for tissue engineering. The development of iPSCs was awarded the 2012 Nobel Prize in Medicine and Physiology, and these iPSCs have largely replaced many ESCs
- Pluripotent stem cells include embryonic stem (ES) cells derived from inner cell mass of the early embryo, embryonic carcinoma (EC) cells derived from undifferentiated primordial germ (PG) cells, or embryonic germ (EG) cells from the late embryonic or early fetal period.
- Organoids, exosomes, and cell extracts from a wide variety of stem cells are actively studied for in vitro and in vivo applications.
- Stem cells exhibit normal growth control, but cancer stem cells represent the uncontrolled growth of mutant cells that can have a negative effect in the body. Steps to prevent any deleterious cells arising in a stem cell population are commonly part of therapeutic process development.

Classical experiment

The most famous animal created by somatic cell nuclear transfer would be Dolly the sheep. She was created in the late 1990s by taking the nucleus from an adult mammary cell of a 6 year old Finn Dorset sheep and placing this nucleus into the cytoplasm of an enucleated egg cell of a Scottish Blackface ewe and brought to full term birth in another Blackface ewe surrogate. The offspring was named Dolly and has only the genes of the Finn Dorset sheep and their typical white face. Dolly was produced at the Roslin Institute in Edinburgh, Scotland, under the leadership of Ian Wilmut with a team that included embryologists, surgeons, veterinarians, and animal care technicians. There were previous offspring of sheep produced at the Roslin Institute by using embryonic cell nuclei, but Dolly was the first produced using an adult nucleus from a differentiated tissue source. This was proof that the adult nuclei could be reprogrammed to totipotency by the cytoplasm of an egg. Dolly was healthy and mated normally, producing six healthy lambs over her 6 years lifespan at the Institute. She developed several normal ailments in her lifetime and was euthanized in 2003 due to tumors in her lungs (Fig. 2.30).

Since this development, cloning of nonhuman primates have been reported.

Immune rejection is another concern that can be expected from any human ES cell line that is not derived from the patient's own cells. Like patients with organ transplants, patients injected with human stem cells may have to take immuno-suppressive drugs for the rest of their lives. ES cells could be genetically modified to escape host immune responses. Alternatively, use of patient-specific iPS-derived cells is a possible solution. However, it has also been shown that iPSCs transplanted back into the same strain of inbred mice elucidated a similar immune response as ES cells derived from the same strain. Thus, it is currently unclear whether patient-specific iPS transplants will solve the problem of immune rejection.

Another challenge will be the purification of differentiated cell types from the heterogeneous cell populations that normally result from most current methods of differentiation. The complex signaling pathways and growth factor requirements to differentiate ES and iPS cells into mature functional cell types are not fully understood. As a result, even though

Dolly: The Cloning of a Sheep, 1996

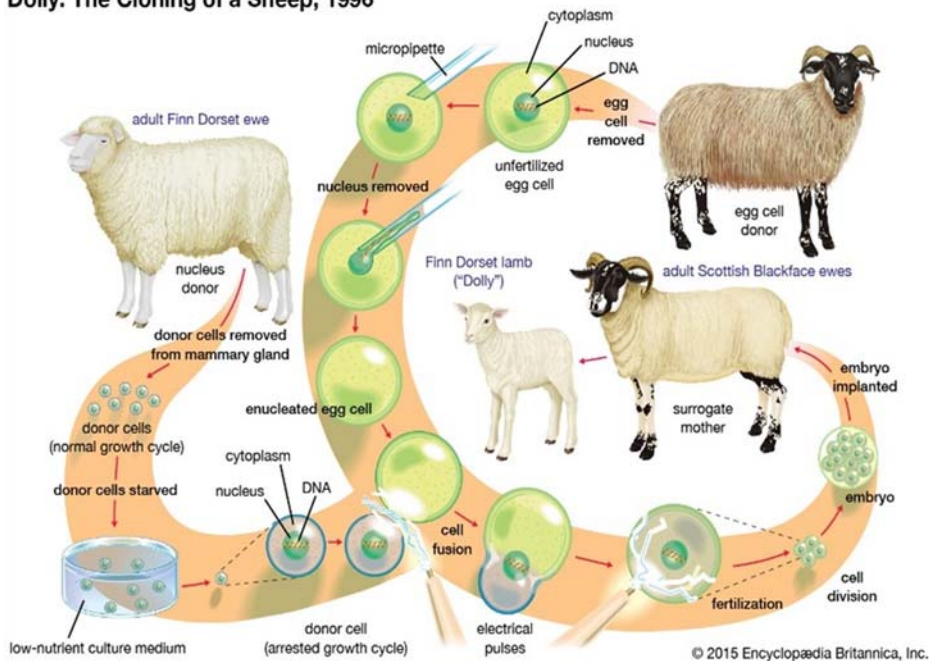


FIGURE 2.30

The process of Dolly, a female Finn-Dorset sheep, resulting in the world's first successfully cloned mammal in 1996. From Judith L. Fridovich-Keil. *Dolly the sheep; cloning*. Encyclopædia Britannica, 2021. <https://www.britannica.com/topic/Dolly-cloned-sheep#/media/1/866570/60421>.

Classical experiment—continued

specific growth factors are added to the culture medium, the outcome is usually a low proportion of differentiated cells in a mixed population of cells. Purification of a cell population can be done by selecting for tissue-specific surface markers, or by transducing the cells using a tissue-specific promoter to drive a selectable marker such as antibiotic resistance. Using the latter method, only cells of the desired tissue type will survive treatment with antibiotics.

Besides selecting for the differentiated cells, it will also be important to negatively select for the remaining undifferentiated population of pluripotent stem cells. These cells, once implanted into the patient, may have the capacity to form teratomas. Although these tumors are generally benign, it is a highly undesired side-effect.

Another major milestone will be the demonstration of treatment efficacy in large animal models. ES and iPSCs have been isolated from nonhuman primates, which are widely used as experimental animal models for human diseases. These pluripotent stem cell lines could be a useful resource for preclinical stem cell research and developing ES and iPSC-based transplantation therapies (Fig. 2.31).

Over recent years, there has been tremendous progress in the isolation procedures and differentiation of human pluripotent stem cells, but therapeutic application is still premature. The first clinical trial using human pluripotent stem cells involved human embryonal carcinoma cells differentiated into neural cells to treat stroke. While this study showed significant signs in patient recovery early on from transplantation and no adverse effects, it did not show long-term benefits.

As of this writing in mid-2022, there were 68 clinical studies listed on the clinicaltrials.gov Website utilizing ESCs of which 18 were recruiting, enrolling or active for a diverse group of conditions or diseases including amyotrophic lateral sclerosis, age-related macular degeneration, retinal pigment epithelial cells, and others. Many of these studies are differentiating the ES cells before transplantation.

Wilmut I, et al. Viable offspring derived from fetal and adult mammalian cells. *Nature*. 1997; 385(6619):810–813. (Original Dolly paper).



FIGURE 2.31

Dolly and the egg cell donor. *From Dolly and her surrogate mother. The life of Dolly, Roslin Institute.* <https://dolly.roslin.ed.ac.uk/facts/the-life-of-dolly/index.html>.

State-of-the-art experiment

Can we cure diabetes with iPS cells

Although treatment is widely available, diabetes remains a major human disease with many comorbidities and the number of patients is rising worldwide with the aging population. Type I diabetes is caused by the autoimmune-mediated loss of pancreatic beta cells and is the disease form for over 450 million people worldwide. It requires daily insulin injection. Type II diabetes is insulin/beta cell insufficiency, occurs in many older individuals, and often can be treated without injection. Currently, human insulin for daily injection is produced commercially in bio-engineered yeast or *Escherichia coli* bacteria. The insulin is purified, free from yeast or bacterial by-products and is administered by injection or refillable indwelling insulin pumps. Another approach that has had success for a limited number of patients is cadaveric pancreatic islet transfer, but this requires the islets from several cadavers and life-long immunosuppression.

New studies suggest insulin producing cells derived from human iPSCs that could be implanted may provide an alternative to daily injections. Patient biopsies are taken, cells isolated, and reprogrammed into iPSCs and then differentiated to iPSC-derived islet cells that contain insulin producing β -cells. These are isolated from the nonreprogrammed cells and provide the working cells necessary to develop the clinical therapy for diabetics (Fig. 2.32). Still, there are several steps that need optimization before delivery back to the patients. There is also the approach to correcting a diabetes-causing monogenic DNA lesion using CRISPR/Cas9 and isolating the corrected cells for reimplantation into patients. Diabetes is a good disease to prove the value of iPSC technology and could provide a working example for development of many other disease therapies using iPSC therapy.

Bourgeois S. et al. Toward a functional cure for diabetes using stem cell-derived beta cells: Are we there yet? *Cells*. 2021;10(191):1–24. <https://doi.org/10.3390/cells10010191>.

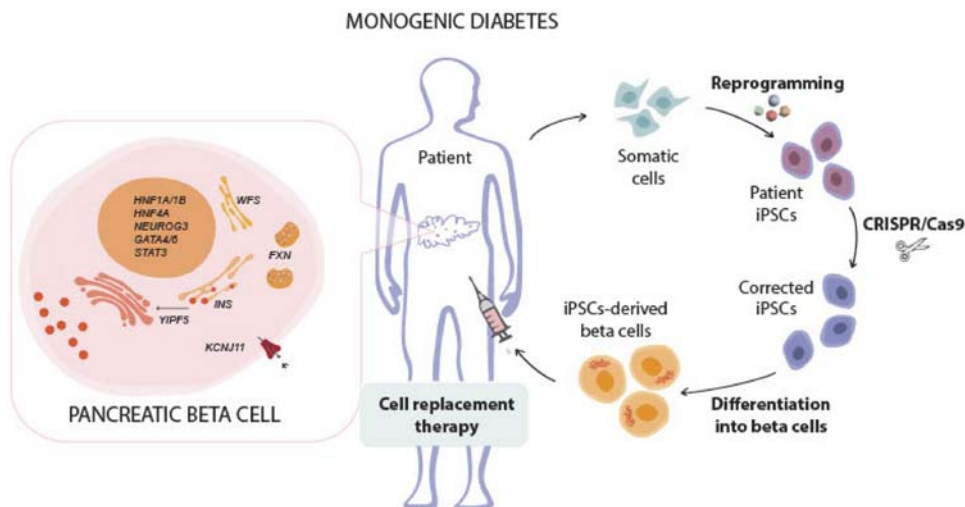


FIGURE 2.32

The promising technique of using engineered human iPSC-derived beta cells for monogenic diabetes. *From Toward a functional cure for diabetes using stem cell-derived beta cells: Are we there yet?*, Bourgeois S. et al. *Cells* 2021, 10, 191. 1–24. <https://doi.org/10.3390/cells10010191>.

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2.25 Assessment of your knowledge

- a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter.
 1. In the early embryo, what are the three layers of cells from which other cells and tissues are derived?
 2. What abilities define a stem cell?
 3. Stem cell division may be _____ or _____.
 4. A billion cells are the result of 1 cell undergoing _____ cell divisions. (number)
 5. In developing a cellular product, the tissue engineer will need to develop an assay of _____, while the doctor or company involved in developing the product will need to develop a _____ assay.
 6. Many tissues in the adult may have reserve stem cells that are in a nondividing state called _____.
 7. Cell death is a normal occurrence and occurs by what four processes:
 8. Many cells require attachment to a substrate or an extracellular matrix. Otherwise, cell death occurs by the process termed _____.
 9. Analyzing the surface molecules on cells is most often done by using fluorescent antibodies and flow cytometry, also known as _____. (abbreviation)
 10. A stem cell colony derived from a single cell may exhibit heterogeneity of gene expression within those cells over time. This is called _____ differentiation and will be followed by stepwise _____ differentiation.
 11. DNA sequencing methods have been adapted to analyze the RNA complexity of a cell by first isolating the RNA, and then converting it to its complementary DNA using reverse transcriptase. Then the many cDNAs for study undergo automated sequencing. This method is known as _____.
 12. Epigenetic modifications of DNA can regulate gene expression by restricting the access of transcription factors to their DNA binding sites. This type of chromatin remodeling usually involves the common epigenetic modifications 1) _____ and 2) _____.
 13. Canonical intercellular signaling by Wnt utilizes _____ as its intracellular second messenger to alter gene expression.
 14. Hematopoietic stem cells can produce all blood-derived cell types but are (easy/difficult) to produce with current in vitro conditions. They are the (most/least) utilized stem cell in clinical therapies.
 15. Mesenchymal stem cells, also called mesenchymal stromal cells, or simply MSCs are most commonly isolated from _____ and _____.

16. MSCs can be readily differentiated in vitro into specific lineages with >95% of the MSCs becoming either _____, _____, or _____, depending on the specific conditions.
 17. The MSCs produce multiple _____ and _____ that enhance tissue repair and _____ the immune response.
 18. The skin has different stem cells in the _____ layer and in the _____.
 19. The intestinal epithelium has one of the most rapid turnovers of all tissues. Its stem cells are found in the _____ and may be identified by expression of _____ or _____.
 20. The reprogramming of somatic fibroblasts to induced pluripotent stem (iPS) cells was first accomplished with viral vectors overexpressing the four genes _____, _____, _____, and _____.
 21. To produce iPS cells without integrating viruses, three strategies are being tested: 1) _____, 2) _____, and 3) _____.
 22. The central nervous system has three sites known to produce more neural cells. These sites are known as the _____ zone, _____ and _____ bulb.
 23. Pluripotent stem cells from mice have proven very useful. In a _____-_____ mouse, the gene product is not produced, and the biological function of the gene can be inferred. Similarly, a _____-_____ mouse can be genetically engineered to over produce a gene of interest, or a _____-_____/_ mutant can be created in which the gene product can be turned on or off.
- b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations.
1. What conditions can influence whether stem cells undergo symmetric or asymmetric division?
 2. Draw the cell cycle and label important stages.
 3. Why would apoptosis be a necessary biological process?
 4. How would you analyze the molecules on the surface of stem cells?
 5. How would you analyze the differentiation potential of isolated stem cells?
 6. How would you determine that your stem cells in a dish are not a collection of somatic cells with different differentiation potential?
 7. Making the first iPS cells required using constitutive overexpression of four gene products. What limitations does this impose? (Hint: Why are these original iPS cells not used in clinical trials?)
 8. What do you think are the properties of cancer stem cells that differentiate them from other stem cells?
 9. How would you isolate a new stem cell?

Challenge-based learning

The use of iPSCs to model SARS-Cov-2 infection in kidney organoids

Note for teachers: A CBL user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Severe acute respiratory syndrome (SARS) is caused by a novel microorganism called coronavirus 2. The resulting coronavirus disease (COVID-19) is an infectious problem caused by the SARS-Cov-2 virus which has unleashed a

Challenge-based learning—continued

pandemic never seen before. Over the past 3 years, this disease caused seven million casualties worldwide together with incalculable societal and economic consequences. The infection mechanism of SARS-Cov-2 virus begins by adhering to ACE receptors in epithelial cells of the upper respiratory tract. Uncontrolled disease can generate acute respiratory distress and multisystemic organ failure affecting the brain, heart, liver and kidney. In many COVID-19 patients, there is a need to control not only the inflammation in the lungs but also fibrosis in the kidney. Disease platforms to model and understand kidney fibrotic mechanisms after SARS-Cov-2 viral infection are lacking. The vision for such platforms is to accelerate the testing and manufacturing of therapeutic tools to mitigate kidney fibrosis in COVID-19 patients.

Motivation and stakeholders

iPSCs are a subset of stem cells obtained from, e.g., the skin or blood, that have been reprogrammed back to an embryonic-like state. iPSCs can be used to generate different types of cells found in the kidney. These cells can aggregate and form more complex tissue structures named organoids, which are versatile and robust enough to represent kidney's microarchitecture and function. Kidney organoids exhibit filtration and detoxification functions, making them adequate candidates to study the nephropathology of COVID-related fibrosis. Solutions to produce a screening model to study COVID-induced kidney fibrosis should consider the needs, requirements and regulatory, financial and technical boundary conditions defined by stakeholders such as COVID-19 patients, patients with chronic kidney disease, nephrologists, critical-care physicians, and microfabrication bioengineers.

Problem definition

Kidney organoids contain different functional cell types that can be found in normal kidney but their spatial organization does not reflect normal kidney architecture. Kidney fibroblasts are not normally included in kidney organoids because fibroblasts result from the differentiation of kidney organoid cells after SARS-Cov-2 stimulus. Therefore, there are no tissue engineered kidney organoids

that reflect the kidney's complex architecture and function, and that drive a specific subset of organoid cells to deposit scar tissue to mimic fibrosis after SARS-Cov-2 infection.

Challenge

To generate a kidney organoid with the same anatomy and architecture as native human kidney and to guide the transition of organoid cells to fibroblasts after a viral exposure of SARS-Cov-2.

Learning framework

Reading the Stem Cells chapter and related literature relevant to the challenge will help you to understand:

1. The anatomy and physiology of the kidney.
2. The pathophysiology and progression of chronic kidney disease.
3. The methods and tools to generate iPSCs from somatic cells.
4. The state-of-the-art clinical strategies to mitigate kidney fibrosis.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

5. The protocols to deliver viruses into kidney organoids.
6. The engineering strategies to fabricate organoids (integrate cells, materials, growth factors, and biomolecules to the fabrication strategies).
7. The strategies to induce fibrosis in kidney organoids.
8. Molecular markers to validate organoid kidney models subjected to COVID-19 infection.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

2.26 Glossary

Allogeneic is tissue, organ, or cell derived from antigenically dissimilar individuals from the same species.

Apoptosis is a form of programmed cell death that occurs in multicellular organisms.

Asymmetrical division refers to cell division where one daughter cell acquires a different cell fate than the other daughter.

Autologous refers to cells or tissue obtained from the same individual.

Autophagy is the natural, conserved degradation of the cell that removes unnecessary or dysfunctional components through a lysosome-dependent regulated mechanism.

Blastula is an early stage of embryonic development characterized by repeated cell division of the fertilized egg to form a fluid-filled cavity surrounded by cells.

Clinical potency is the concentration or amount of the drug required to produce a defined effect.

Colony forming units-fibroblastic (CFU–F) is a unit used to estimate the number of viable fibroblasts cells in a sample.

Contact inhibition is a property of normal cells where growth of the cells in a culture eventually stops in a cell density-dependent manner.

Differentiation is a transition of a cell from 1 cell type to another and it involves a switch from one pattern of gene expression to another.

Ectoderm is the outermost of the three primary germ layers in early embryonic development. It originates from the outer layer of germ cells and is the precursor of the skin and neural system and associated organs like the eye and the neural crest.

Embryonic stem cells are pluripotent stem cells derived from the inner cell mass of a blastocyst, an early-stage preimplantation embryo.

Endoderm is the innermost of the three primary germ layers in early embryonic development and is the precursor of organs such as the gastro-intestinal tract.

Epigenetic modifications of DNA is a field of genetics that studies the influence of the reversible heritable changes in gene expression that occur without changes in the sequence of the DNA in the cell nucleus.

Exosomes are microscopic extracellular vesicles with a diameter of 30–100 nm, secreted into the intercellular space by somatic cells.

Fluorescence activated cell sorting (FACS) is a specialized type of flow cytometry, based upon the specific light scattering and fluorescent characteristics of each cell. Used for sorting a heterogeneous mixture of biological cells into two or more containers, 1 cell at a time.

Hematopoietic stem cells (HSCs) give rise to different types of blood cells, in lines called myeloid and lymphoid.

Homeostasis is the state of steady internal, physical, and chemical conditions maintained by living systems.

Immune suppression is a reduction of the immune system activity which can be accomplished artificially via radiation, medications, surgeries, or plasmapheresis or a disease.

Induced pluripotent stem cells are a type of pluripotent stem cells generated directly from a somatic cell by expressing the transcription factors Oct 4, Sox2, Klf4, and c-Myc.

Inner cell mass is the mass of cells inside the primordial embryo that will eventually give rise to the definitive structures of the fetus.

Knock-out mouse is a genetically modified mouse in which an existing gene has been inactivated by replacing or disrupting it with an artificial piece of DNA.

Mesoderm is the middle of the three primary germ layers in early embryonic development and precursor to among others muscle and connective tissue.

Necroptosis is an alternative mode of regulated cell death mimicking features of apoptosis and necrosis.

Necrosis is unprogrammed cell death due to cellular damage or infiltration by pathogens, as opposed to orderly programmed cell death via apoptosis.

Pericytes are multifunctional mural cells that wrap around the endothelial cells lining the capillaries throughout the body. Their contraction and relaxation affect the shape of blood vessels.

Potency is the ability of cells to differentiate to multiple lineages.

Progenitor cells can differentiate into a specific type of cell but is already more lineage restricted than a stem cell.

Quiescence is a state of a cell when it is not dividing and metabolically inactive.

Regenerative medicine is actively enhancing the body's ability to heal tissues by understanding and intervening in the repair process of damaged or diseased tissue to achieve better outcomes.

Self-renewal is the ability to go through numerous cycles of cell growth and cell division while maintaining the undifferentiated state.

Stem cell niche refers to a microenvironment within a specific anatomic location where stem cells receive the proper signals to maintain their stem cell properties.

Stem cells are undifferentiated or partially differentiated cells that can differentiate into various types of cells and proliferate indefinitely to produce more of the same stem cell.

Symmetrical division refers to cell division where both daughter cells acquire the same fate as the parent cell.

Temporal stochasticity is the event in the life history of a cell that defines its future, and sometimes that of neighboring cells.

Transit amplifying cells are an undifferentiated population of cells undergoing cell division to become differentiated cells.

© Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

2.27 Further reading

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Tissue formation during embryogenesis

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3.1 Learning objectives

After reading this chapter you will be able to:

- Appreciate that tissue engineering strategies rely on the merger of developmental biology with the fields of engineering in a strategy nowadays known as developmental (re)engineering.
- Understand the origin of pluripotent stem cell populations in the embryo and to realize that each organ is build out of stem cells from endodermal, mesodermal, ectodermal, and neural crest origin.
- Recapitulate some of the basic principles by which cellular differentiation and specification is induced during organogenesis.
- Appreciate that the subsequent, timely and proper dosing of signaling events, for example, initiated by cell migration, cell–cell interactions, para- and autocrine signaling of growth factors, epithelial-to-mesenchyme transitions, and environmental factors define cell specification and shape organs.
- Realize that key concepts from developmental biology such as modularity and robustness can be recapitulated in engineering strategies to generate functional organs.

Some well-accepted empirical concepts of developmental biology, such as path-dependence, robustness, modularity, and semi-autonomy of intermediate tissue forms, that appear sequentially during tissue development are starting to be incorporated in process design, named developmental engineering.

P. Lenas, 2009.

How superior to our mind is nature's own experiment.

L. Vroman, 1992.

3.2 Introduction

The goal of this chapter is to provide the reader with a basic introduction in some of the principles of tissue and organ formation during embryonic development. Insights in the origin of (stem) cells in the **71**

embryo and the mechanisms that drive cell differentiation, tissue formation, and maturation are directly relevant to design a successful tissue engineering strategy. This chapter aims at providing a background on the formation of various organs during embryogenesis that are subject of tissue engineering techniques, like the heart, blood vessels, peripheral nerves, skin, bone, and cartilage. It starts with providing an overview of the formation of the three germ layers, the ectoderm, mesoderm, and endoderm during gastrulation and the formation of highly migratory and plastic neural crest cells (NCCs) and their positioning in the overall body plan. Subsequently, some of the basic mechanisms by which these cell populations are recruited in organogenesis and are induced to specify are discussed in greater detail. At various places, direct links are made with currently used tissue engineering strategies.

3.2.1 Organ formation during embryogenesis

Organs and tissues in the human body are the result of millions-of-years of evolution. This process has resulted in the natural selection of organs that are optimally adapted to their function in the body. Almost all adult tissues, with a few exceptions like for example the heart, are capable of self-repair after (limited) injury by activating intrinsic repair mechanisms. These intrinsic repair mechanisms recapitulate many of the processes involved in the formation of an organ during **embryogenesis**. Not surprisingly, tissue engineers try to mimic these processes in vitro to engineer functional tissue as well. Thus, important lessons for tissue engineering can be learned from the formation of organs during embryogenesis. For example, on (1) the origin of (stem) cells that contribute to the formation of a particular organ, which helps in selecting the most relevant cell type for tissue formation; on (2) the sequential use of growth factors and other signaling molecules and their interrelationship in subsequent steps of cell differentiation, which helps in choosing the right set of growth factors for engineering a specific tissue; on (3) the role of cell–cell interactions and/or cell migration in tissue formation which helps in selecting the right combination of cells for engineering of multicellular tissues which has proven critical for achieving tissue maturation; on (4) the role of environmental factors like exposure to air, mechanical forces, and/or exposure to variations in oxygen tension in cell differentiation and tissue maturation, which helps in optimizing culture conditions; and on (5) genetic markers that are characteristic for subsequent stages in stem cell differentiation, which allow to examine progression in the development and/or tissue maturation of tissue engineered constructs ([Table 3.1](#)). Ideally, inclusion of all these

Table 3.1 Tissue engineering lessons to be learned from embryogenesis.

1	Origins of (stem)cells that contribute to tissue formation
2	Selection of relevant growth factors and other stimuli for differentiation
3	Regulatory mechanisms involved in multistep differentiation pathways
4	The role of cell–cell interactions in differentiation
5	The role of cell migration in formation of specific tissue structures like blood vessels and peripheral nerves
6	The role of environmental factors like oxygen levels and mechanical forces in tissue maturation
7	Selection of markers to monitor differentiation and tissue maturation

aspects in the tissue engineering procedure will provide the best opportunities for success. Indeed, one of the basic concepts of developmental biology, i.e., the **modularity of the tissue architecture** according to which intermediates in tissue development constitute semiautonomous entities which are subsequently combined to form larger tissue structures, is nowadays incorporated in many tissue engineering strategies to optimize the product in so-called bottom-up tissue engineering strategies.

The past decade has shown a rapid increase in our understanding of the cellular and molecular basis of tissue formation. In addition, our knowledge about stem cell renewal and maintenance of pluri- and multipotency has substantially grown. It still remains a challenge to combine this knowledge into successful strategies for the engineering of functional tissues that can replace lost or worn-out organs. The tissue engineering strategy in which the principles of developmental biology are merged with typical engineering disciplines like chemical, biomechanical, and biophysical engineering is nowadays known as **developmental engineering** or developmental (re)engineering.

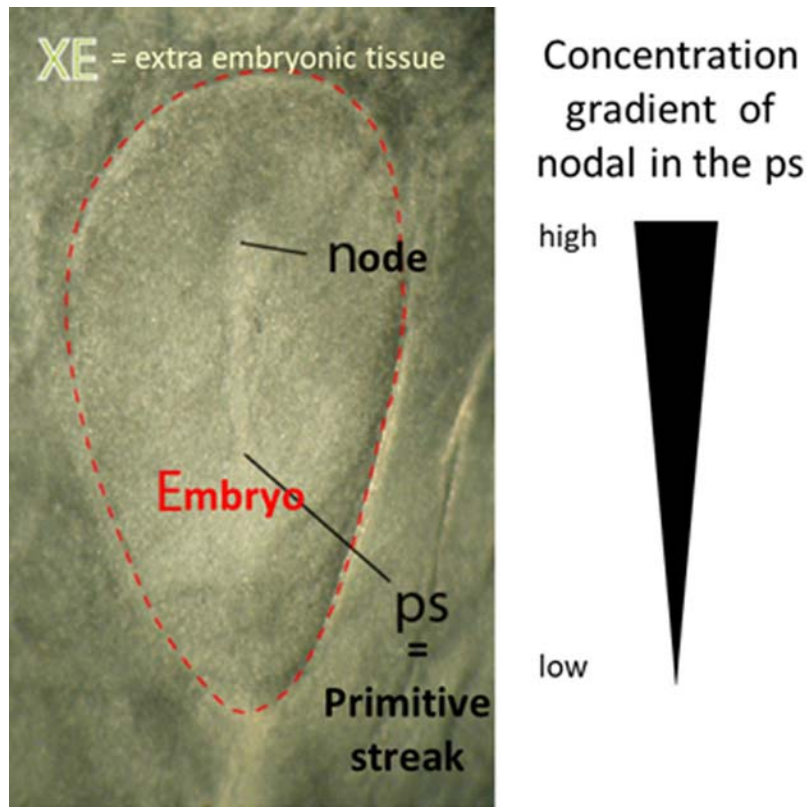
3.2.2 The formation of the three germ layers during gastrulation

Shortly after implantation of the developing embryo in the uterus wall, a process called **gastrulation** starts. In this process, pluripotent cells of the inner cell mass of the blastocyst reorganize and specify in one of the three germ layers, the ectoderm, endoderm, and mesoderm. The use of pluripotent cells from the inner cell mass to establish embryonic stem cells (ES) is discussed in [Chapter 2 Stem cells](#). The derivation of these cells is described in the text box of the classical experiment. This chapter also discusses the derivation of induced pluripotent stem cells (iPSCs) by dedifferentiation of mature tissue cells using the forced overexpression of reprogramming transcription factors like OCT4, KLF4, cMYC, and SOX2. These iPSCs are functionally equivalent to ES cells.

During gastrulation, the three definitive germ layers that give rise to the adult organism are established: the outer ectoderm, the inner endoderm, and the interstitial mesoderm. The **ectoderm** forms the outer part of the skin, brain cells, nerve cells, parts of the eye like the lens, epithelial structures of the mouth and anus, the pituitary gland, parts of the adrenal glands, and pigment cells. The **endoderm** forms the lining of the gastrointestinal and respiratory tracts, plus the liver, pancreas, thyroid gland, thymus, and the lining of the bladder. The **mesoderm** gives rise to skeletal muscle, heart and blood vessels, connective tissue, kidney, urethra, gonads (but not germ cells), bone marrow, blood, bone, cartilage, and fat. During gastrulation, extensive cell movements take place through which cells acquire new positions and new neighbors with which to interact. Replicating cell movement in tissue engineered constructs remains still challenging.

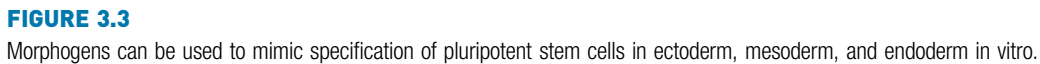
3.2.3 Establishment of the body plan by morphogen signaling

During gastrulation, not only are the germ layers specified but cells will also receive instructions on their future position and role in the developing fetus by morphogen signaling. **Morphogens** are classes of growth factors that help establish the **body plan**. An example of such morphogen is Nodal, which is a member of the Bone Morphogenetic Protein (BMP)/Transforming Growth Factor β (TGF β) superfamily of growth factors. Nodal signaling is essential for the formation of mesoderm and definitive endoderm. The highest levels of Nodal are found in the node, a structure which orchestrates gastrulation, and with increasing distance to the node, the concentration of Nodal will gradually decrease establishing a so-called morphogen gradient ([Fig. 3.1](#)).

**FIGURE 3.1**

The node orchestrates formation of mesoderm and endoderm by morphogen signaling.

Cells along this gradient are exposed to different levels of Nodal, providing cells with distinct instructions on its future position and role in the fetus. Morphogen gradients are important and recurrent mechanisms by which undifferentiated cells receive instructions on their future position and role in the body. Other morphogens that play a role in specification of cells during gastrulation are, besides Nodal, other members of the TGF β /BMP superfamily of growth factors like TGF β 1 and 2, BMP2 and 4, and Activin A. Also, members of the Wingless Integrated (WNT) growth factor family and basic Fibroblast Growth Factors (bFGF) play an important role (see [Chapter 4 Cell signaling](#)). Either activation of the respective signaling pathways by gradients of growth factors or inhibition of growth factor activity by gradients of specific growth factor antagonists is used to specify cells during gastrulation. The gradients of growth factors or growth factor antagonists are often reciprocal ([Fig. 3.2](#)). Using the knowledge of morphogen signaling during gastrulation, it is now possible to replicate this process in vitro by treating pluripotent ES or iPS with combinations of these factors to obtain endodermal, mesodermal, or ectodermal cells which subsequently can be used for further differentiation into respective tissues ([Fig. 3.3](#)). Most of the growth factors used in tissue engineering strategies act as morphogens during embryonic development.



Besides the three germ layers that are established during gastrulation, a fourth highly plastic cell population is required for **organogenesis**. This population consists of NCCs. Although NCCs are derived from the ectoderm, they are sometimes called the fourth germ layer because of their importance. NCCs arise relatively late in embryonic development after establishment of the general body plan and their formation is tightly linked with the development of the central nervous system. The differentiation of the central nervous system starts with formation of the neural tube from the neural plate. The neural

plate is a layer of ectodermal cells in the **dorsal** midline of the embryo. Signals from the underlying notochord, a mesodermal structure that will develop into the future **nucleus pulposus** in the vertebral column, instruct cells in the neural plate to proliferate and adapt a tube-like shape that will eventually generate the entire central nervous system. NCCs are generated at the interface of the neuroectoderm with the surface ectoderm, the so-called neural plate border (Fig. 3.4). It is thought that the surface ectoderm together with the underlying mesoderm induces the neural plate to form NCCs by signals that include low levels of BMP signaling, in particular BMP4 and BMP7, FGFs, and members of the WNT family of morphogens.

From their source of origin, NCCs migrate to specific places in the embryo where they generate a diverse group of differentiated cells that can be divided into four distinct subgroups: cranial, cardiac, vagal, and trunk cells that together give rise to sympathetic and parasympathetic neurons, glia cells, fat, cardiac mesenchyme, melanocytes, skin, connective tissue of salivary, thymus, adrenal, thyroid, and pituitary glands, smooth muscle cells (SMCs) of arteries, tooth, and bone and cartilage particularly of the face and cranium. The NCCs therefore have extreme plasticity and are **pluripotent** giving rise to cells

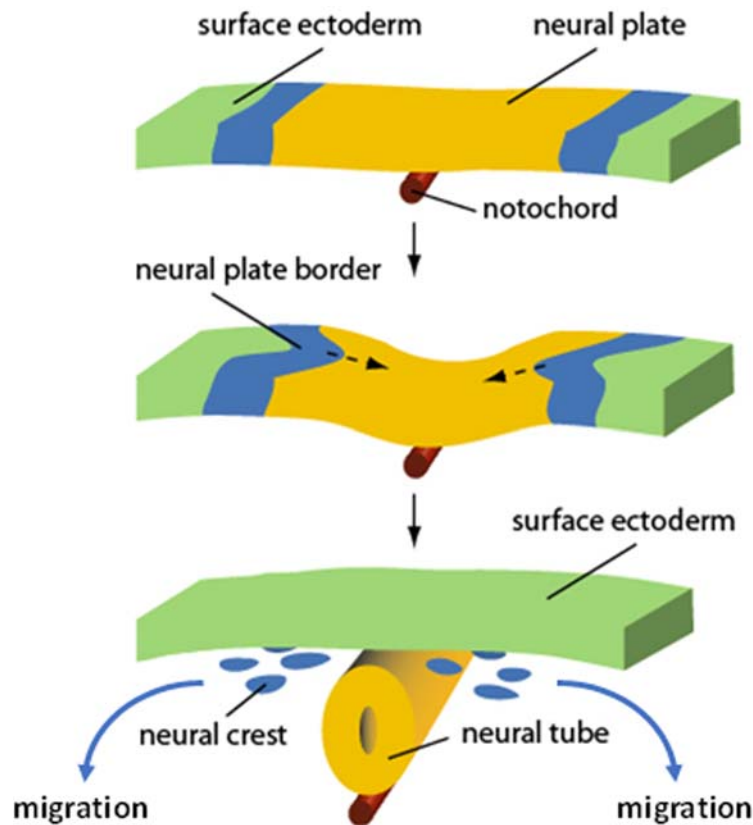


FIGURE 3.4

Migratory neural crest cells arise at the neural plate border.

from the endoderm, mesoderm, and ectoderm lineage. Due to this extreme plasticity, NCCs would provide an excellent cell source for tissue engineering and regenerative medicine particularly for restoration of cranial and facial birth defects. However, NCCs are extremely difficult to isolate due to their high mobility and to the fact that they are derived from a transient structure. The neural crest disappears soon after the neural tube is closed, and the neural plate border has disappeared. Consequently, limited studies have been done with NCC as cell source for tissue engineering purposes and little is known how to maintain NCC in vitro as a self-renewing population.

It is now, however, possible to obtain NCCs in vitro using pluripotent ES or iPS by treating cells with activators of canonical WNT signaling and inhibitors of BMP and activin/nodal signaling. Subsequent treatment with factors like FGF2, retinoic acid, and BMP4 can induce further regional specification of the NCC along the anterior to posterior axis of the embryo (Fig. 3.5).

Since NCCs are derived from the ectoderm which differentiate normally into cells that lack migratory potential, NCCs have to undergo an epithelial-to-mesenchymal transition (EMT) to acquire cell features essential for cell migration. Such features are normally only present in cells of the mesodermal lineage. During an EMT, the epithelial cells lose cell polarity characterized by differences in the **apical** and **basolateral** membrane, and the expression of cell adhesion molecules and proteins involved in the formation of tight junctions which are key characteristics of epithelial cells. EMT is an important mechanism by which cells can dramatically change their fate (Fig. 3.6). It plays an essential role not only in

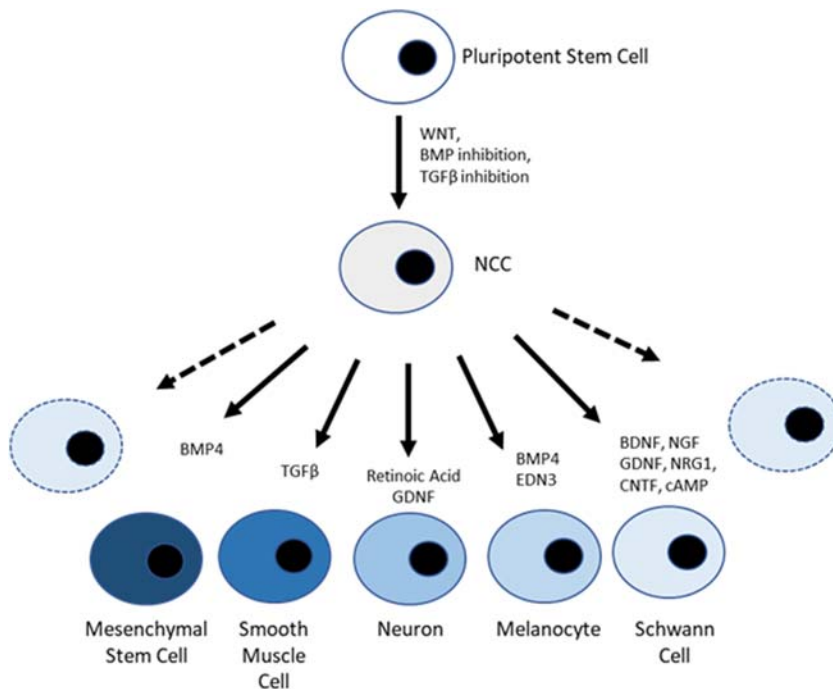
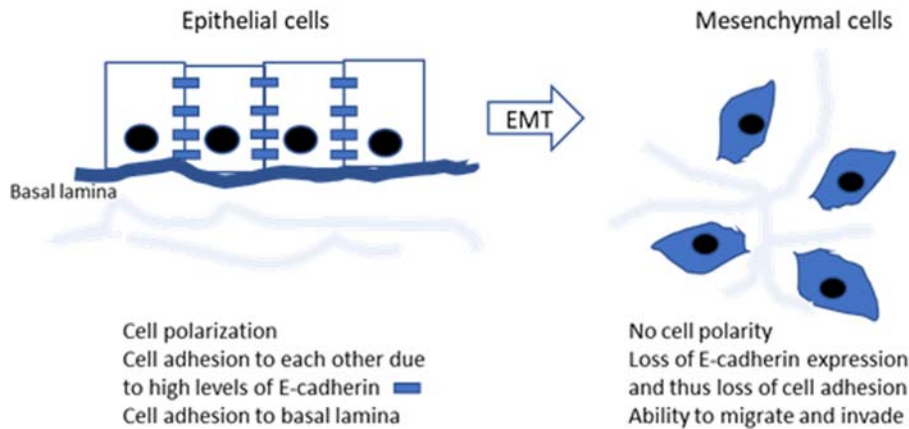


FIGURE 3.5

Derivation of neural crest cells and their further specification from pluripotent stem cells in vitro.

**FIGURE 3.6**

Cells become migratory due to an epithelial to mesenchyme transition.

organogenesis but also during tumorigenesis, e.g., the transformation of a carcinoma in situ to an invasive, metastatic tumor is preceded by an EMT of epithelial tumor cells.

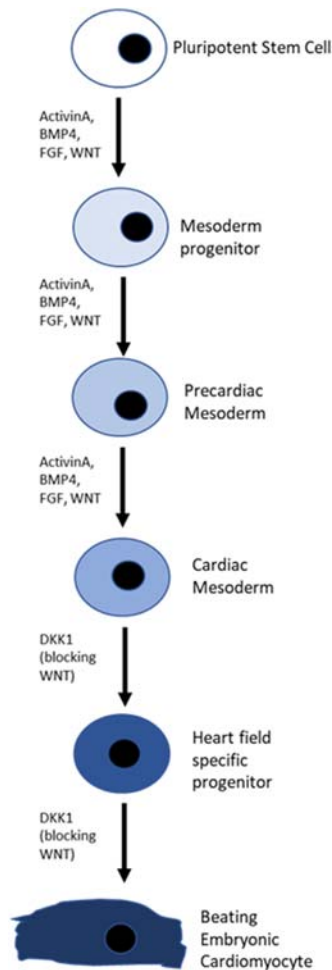
3.3 Cardiac development

Repairing the injured heart after infarction with contracting cardiomyocytes is a major challenge for tissue engineering. Various cell-based approaches are under development (see [Chapter 18 Principles of cardiovascular tissue engineering](#)). Initially, this work started with committed myoblasts, the precursor cells of cardiac muscle, but now a variety of undifferentiated cells are used, such as mesenchymal stem cells (MSCs), resident cardiac stem cells, and most importantly pluripotent ES and iPS. Detailed protocols are now available for the reproducible differentiation ES and iPS in contracting cardiomyocytes ([Fig. 3.7](#)).

The two most important cell sources contributing to the formation of the four-chambered cyclically beating heart during embryonic development are mesodermal cells derived from the so-called cardiogenic plates and the cardiac NCC ([Fig. 3.8](#)). The cells of the cardiogenic plates give rise to the contracting myocardium and the endocardium. Cells of the cardiac NCC give rise to the arterial vessel wall, adventitial fibroblasts, and SMCs surrounding the blood vessels and arteries. After cardiac infarction, the fibroblasts provide for the scar tissue giving integrity to the cardiac wall, although it is, of course, not contractile. However, in early embryonic development, the fibroblasts are responsible for the induction of the architecture of the cardiac wall.

3.3.1 Geometrical changes transform a single beating heart tube into a four-chambered heart

The beating heart is the first active organ during embryogenesis and must continuously adapt to the growing needs of the embryo. In postnatal life it has to support the continuously changing activity levels of the body. The proper and subsequent integration of all these processes is of crucial importance

**FIGURE 3.7**

The subsequent steps for differentiation of pluripotent stem cells into beating cardiomyocytes.

(Fig. 3.9). Disturbance will result in embryonic heart failure or congenital heart deformities. The development of the heart is modular in which intermediates in tissue development constitute semiautonomous entities. Initially, the heart is formed as a single almost strait tube. By a series of geometrical changes, this tube finally transforms into a double pump, separately serving lungs and body. Such large geometrical changes are still difficult to recapitulate in tissue engineering strategies in the lab. Bio-fabrication technologies such as cell and tissue printing may help in rebuilding complex geometry (see [Chapter 11 Scaffold design and fabrication](#)). The four chambers are separated by valves which arise by a complex interplay between cardiomyocytes and NCC. Replacement of malfunctioning valves, which is a highly relevant clinical problem, by tissue-engineered substitutes is an active field of research.

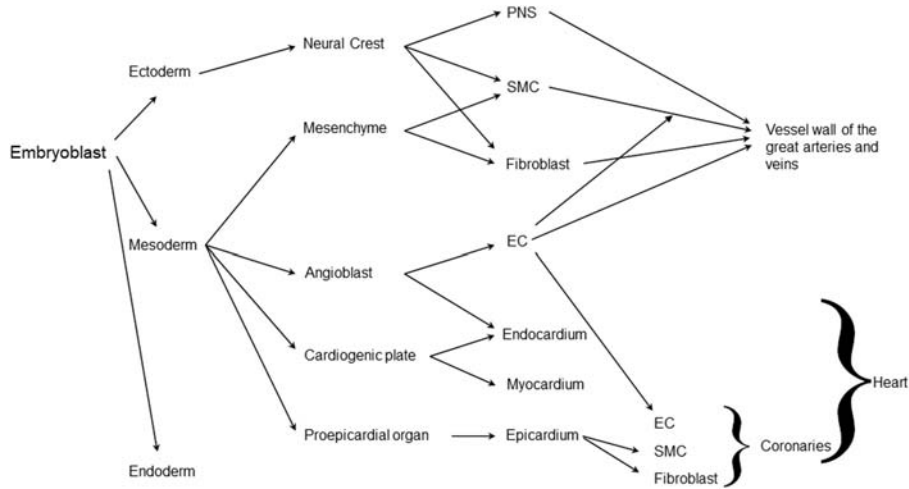


FIGURE 3.8
Overview of the origin of cell sources contributing to heart and blood vessel development.

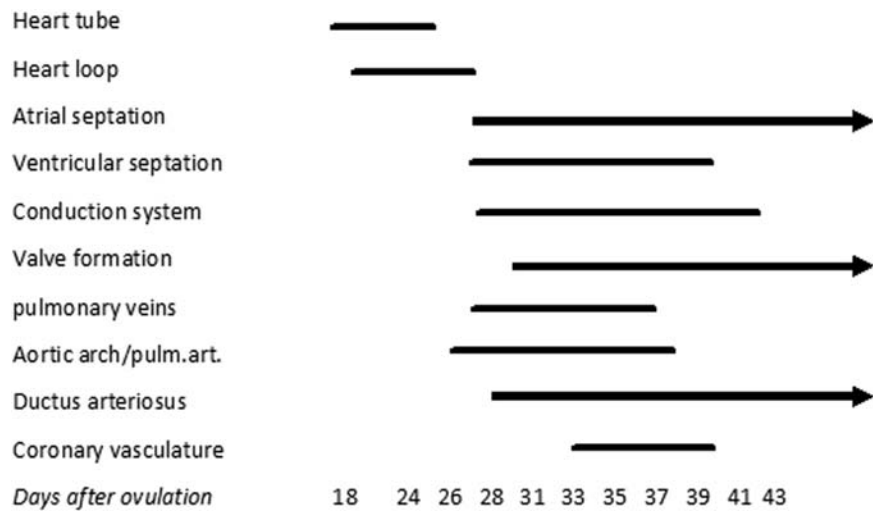


FIGURE 3.9
Timetable of the sequential steps in the formation of the human heart during embryogenesis.

Decellularized valves isolated from porcine or bovine species are frequently used to replace malfunctioning valves in human patients.

This is far from ideal when valves need to be replaced in the growing newborn due to a congenital defect. Growth of the heart would require recurrent replacement surgery of the valves. Transplantation of a cellularized valve which can grow with the developing newborn would solve this issue. The

complexity in the cellular origin of the valves next to their unique biomechanical properties makes such a tissue engineering strategy challenging.

3.3.2 Cardiomyocytes

Tissue engineering strategies to repair the infarcted heart have mainly focused on deriving cardiomyocytes from stem cell sources to engineer beating heart tissue in the lab. Indeed, detailed protocols are now readily available for the differentiation of cardiomyocytes from pluripotent stem cells (Fig. 3.7). The differentiation processes of iPS in beating cardiomyocytes mimics the sequential steps in development of the embryonic heart starting from mesodermal progenitor cells and ending in terminally differentiated cells. However, it is also clear that a single cardiomyocyte phenotype does not exist. Indeed, left and right ventricular, atrial, and out flow tract cardiomyocytes present with a variety of different gene expression patterns in successive time windows which can be explained by the specific demands for cardiomyocytes in each of these tissue structures. It remains challenging to mature the cardiomyocytes into tissue-specific lineages at least in vitro and to obtain fully mature cells. Recent experimental evidence suggests that bringing together various cell types that normally interact with each other in the heart, such as cardiomyocytes, fibroblasts, and endothelial cells, in ratios found in normal heart development may facilitate tissue maturation. This is an example of complex interplay between various cell types that are involved in the formation of a specific organ.

3.3.3 Future perspective

The formation of the heart is a complex, modular, and time-consuming process, which continues during the whole period of gestation and even after birth. Reconstructing and engineering the heart for major diseases must be based on thorough knowledge of the multiple interactions at all levels of development, function, and maintenance of the heart. Rather than engineering a completely new heart, tissue engineering strategies are therefore mainly aimed at reconstituting the infarcted heart with new beating heart muscle. It is now possible to engineer in the lab sheets of cyclically contracting cardiomyocytes derived from pluripotent stem cell sources which, when safety concerns about the use of pluripotent stem cell sources are resolved, could be used for transplantation. Combined with biofabrication technologies complex tissue shapes might be reproduced by cell and tissue printing. However, major challenges are still not resolved, such as cardiomyocyte maturation, the connection with the blood flow, and integration with the conductance system of the heart to ensure synchronous contraction. Also, the differentiation of pulse maker cells orchestrating the cyclic contraction from stem cells and their integration in a tissue engineered heart is still not completely resolved.

3.4 Blood vessel development

A clear need for a suitable arterial replacement has prompted researchers to look beyond autologous and synthetic tissue replacements toward the engineering of vessels using mesenchymal, hematopoietic, and pluripotent stem cells. Besides the development of arterial replacement, incorporation of blood vessels is critical for the successful engineering of any clinically relevant sized tissue for transplantation purposes. Cells and tissues in our body are critically dependent on blood flow for the delivery of

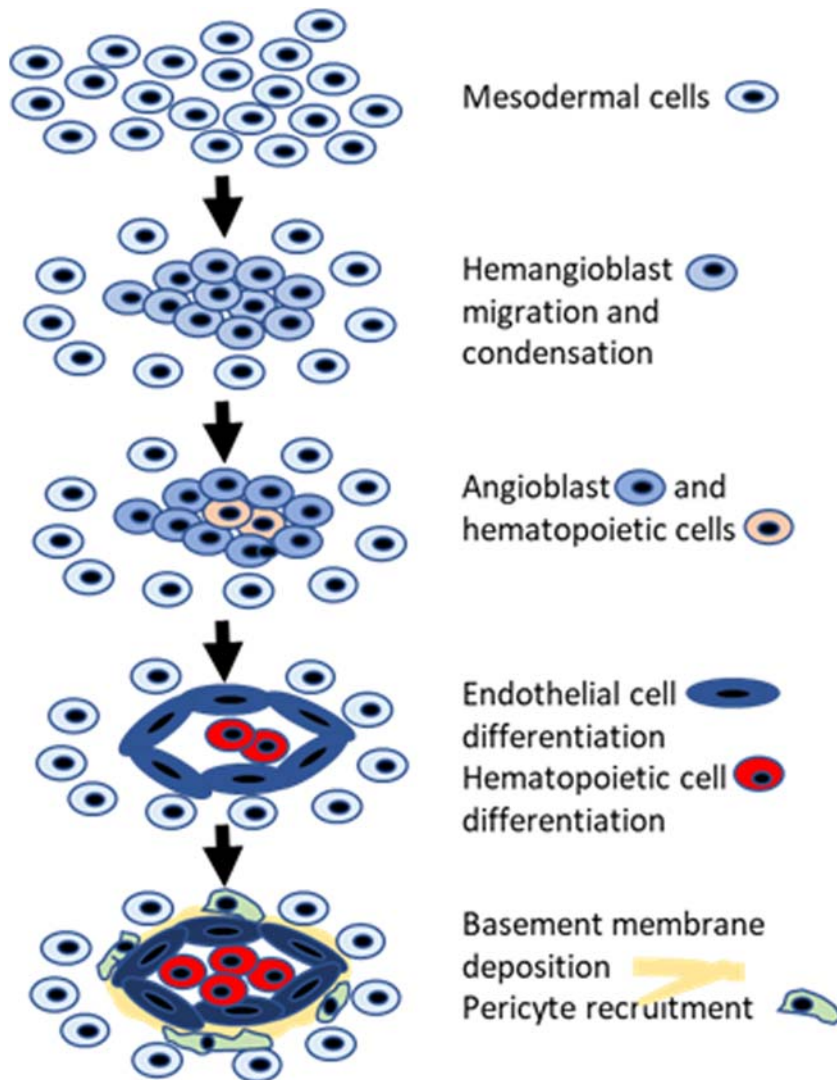
oxygen, nutrients, and the disposal of waste products. Tissues with a volume larger than 100 cubic micrometers cannot survive without a blood vessel. Hence, with few exceptions such as the nonvascularized articular cartilage, engineering of human tissues must go hand in hand with the simultaneous formation of a blood vessel network which needs to be connected to the host blood circulation upon implantation. Lack of blood supply to the inner most parts of a tissue engineered construct is largely responsible for graft failure after transplantation. Also, in the embryo, organ growth is tightly linked to the simultaneous expansion of a vascular network.

3.4.1 Vasculogenesis and angiogenesis

In embryonic development, the formation of the vasculature is tightly linked with heart development. Thus, when the heart tube starts to beat (about 4 weeks after conception in humans), a network of endothelial-lined vessels is in place, through which the blood will be transported to and from the yolk sac. Blood vessel formation starts with the formation of the **angioblast** which is derived from the mesoderm. Cells at the periphery of the angioblast will differentiate into endothelial cells, while the inner cells will give rise to the hematopoietic stem cells (Fig. 3.10). The endothelial precursors line up, connect, lumenize, and form a plexus that gives rise to both the embryonic and extraembryonic vessels in the yolk sac and later in the placenta. This process is called **vasculogenesis**. Vascular endothelial growth factor (VEGF), TGF β , and FGF signaling pathways are key players in the initial establishment of this endothelial network and therefore these factors are frequently used in tissue engineering strategies for blood vessels. Initially, the vasculature formed by the endothelial cells has an almost two-dimensional structure. This plexus will rapidly grow out and gives rise to organ-specific vasculatures within the fast-growing embryo. Outgrowth of blood vessels from this two-dimensional network by sprouting of endothelial cells is called **angiogenesis**. During wound healing angiogenesis drives the formation of new vessels.

3.4.2 Blood pressure drives specification of vessels in arteria or veins

Blood vessels come in a large variety. The high blood pressure in the great arteries sets specific demands for the architecture of these vessels, which are not needed in small arteries penetrating in areas of a particular organ or in the veins which transport the blood back to the heart. This specification of endothelial cells is largely driven by blood flow and blood pressure providing one of the first examples of an environmental cue that drives cell specification in the embryo. Blood flow induces shear stress as well as cyclic expansion and relaxation of ECs. Both processes activate various biochemical pathways. It is known that the expression of many genes is regulated by shear stress inherently linked to fluid flow inside a blood vessel. Flow depends, among others, on the diameter of blood vessels, a larger vessel diameter allowing for more flow. Among many others, the expression of TGF β , endothelial nitric oxide synthase, and VEGF is regulated by shear stress, which subsequently orchestrate tissue architecture, i.e., the alignment of cells and extracellular matrix and recruit auxiliary cells that are needed for vessel stabilization. Indeed, except for the capillaries each vessel will be enveloped by one or more layers of specialized **mural cells**, such as SMCs and pericytes. The origin of these different cell types is presented in Fig. 3.8.

**FIGURE 3.10**

The angioblast gives rise to hematopoietic stem cells and endothelial precursors.

3.4.3 Vessel wall stabilization by smooth muscle cells and pericytes

Vessel wall stabilization critically depends on the recruitment of SMCs for arteries and veins and pericytes for the precapillaries to the preformed endothelial scaffolding. The location of the vessel within the embryo determines the origin of the SMCs (Fig. 3.8). In the greater part of the body, the SMCs have a mesodermal origin. In the head and the thoracic region, the arteries and veins recruit their SMCs from the NCCs. Vascular abnormalities like Marfan (disrupted fibrillin-1), Williams' syndrome

(disrupted elastin), Loeys–Dietz syndrome (TGF β receptor 1 and receptor 2 mutations), and Char syndrome (mutations in the transcription factor AP-2) have preferential sites of occurrence that probably reflect the embryonic origin of the SMCs. The relative abundance of endothelial cells, SMCs, and pericytes largely depends on the position and function of the vessel within the vasculature. Together they give rise to the various types of vessels (muscular and elastic arteries, capillaries, and veins). One has to keep in mind, however, that a single endothelial or a single SMC phenotype does not exist. In fact, there is a large heterogeneity in endothelial and smooth muscle phenotypes reflecting their multiple origins and their site and tissue-specific role in controlling homeostasis of the body.

3.4.4 Recruitment of mural cells is mediated by PDGF signaling

The recruitment of SMCs or pericytes is in part governed by platelet-derived growth factor (PDGF)-B, which is expressed by the endothelial cells. PDGF-B initiates the recruitment of PDGF receptor-expressing mural cells (pericytes and SMCs) toward the newly formed vessel. In turn these cells produce the secreted growth factor angiopoietin 1, which after binding the Tie2 receptor, which is expressed on the endothelial cells, downregulates their angiogenic activities. This is an example of the intensive communication between the endothelial cells and underlying pericytes and SMCs and vice versa (Fig. 3.11). Cross-talk between epithelial or endothelial cells at one hand and the underlying mural cells is a recurrent theme in tissue formation and drives for example the maturation of the lung epithelium, the skin, and the blood vessels.

Pericytes have recently attracted a lot of attention in the field of tissue engineering as they possess stem cell-like characteristics and are considered to be one of the origins of adult mesenchymal stem or stromal cells in the adult organism.

3.4.5 Future perspective

Successful tissue engineering of any vessel in the lab critically depends on the selection of the right combinations of cells. Next to this, environmental factors such as fluid shear stress and pulsatile expansion of the blood vessel by mimicking blood flow need to be considered. For these reasons, preconditioning of blood vessels grown in the lab by simulating pulsatile blood flow through the construct is an essential element in tissue engineering of mature blood vessels. The preconditioning helps in defining the

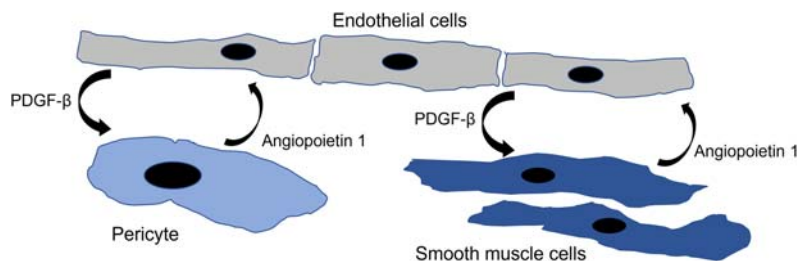


FIGURE 3.11

Cross-talk between endothelial cells and mural cells stabilizes blood vessels.

identity of the endothelial cells. In addition, it helps in the recruitment and the adaptation and orientation of the underlying SMCs, pericytes, and extracellular matrix in order to withstand the mechanical forces exerted by the pulsatile flow. Such tissue maturation process is needed to avoid rupture and hence failure of tissue-engineered constructs shortly after transplantation.

Knowledge of blood vessel formation is also essential for engineering clinically relevant sized tissues and this requests that many tissue engineering strategies need to go side-by-side with a proper vascularization strategy (see [Chapter 14 Vascularization, survival, and functionality of tissue-engineered constructs](#)). Upon implantation, the thus formed vessels need to be connected to the host vasculature upon implantation to ensure graft survival.

3.5 Development of peripheral nerve tissue

Driven by an enormous clinical need, peripheral nerve regeneration has become a prime focus within the field of tissue engineering (see [Chapter 17 Tissue engineering of the nervous system](#)). The various tissue components that make up the peripheral nerve originate from different germ layers. The neurons and glial or Schwann cells are derived from either the neuroectoderm or NCC, while the nerve sheath, which protects the nerves, is derived from the mesoderm. The development and organization of the peripheral nerves with their myelinated and nonmyelinated axonal fibers and their protective epithelial layer involves complex cellular interactions and molecular mechanisms.

3.5.1 Development of the schwann cell lineage

The peripheral nerve contains two types of Schwann cells: myelin-forming Schwann cells that ensheath large caliber axons (diameter larger than 1 μm) and nonmyelinating Schwann cells that accommodate multiple lower caliber axons in cytoplasmic cuffs ([Fig. 3.12](#)). Both cell types are derived from the NCCs

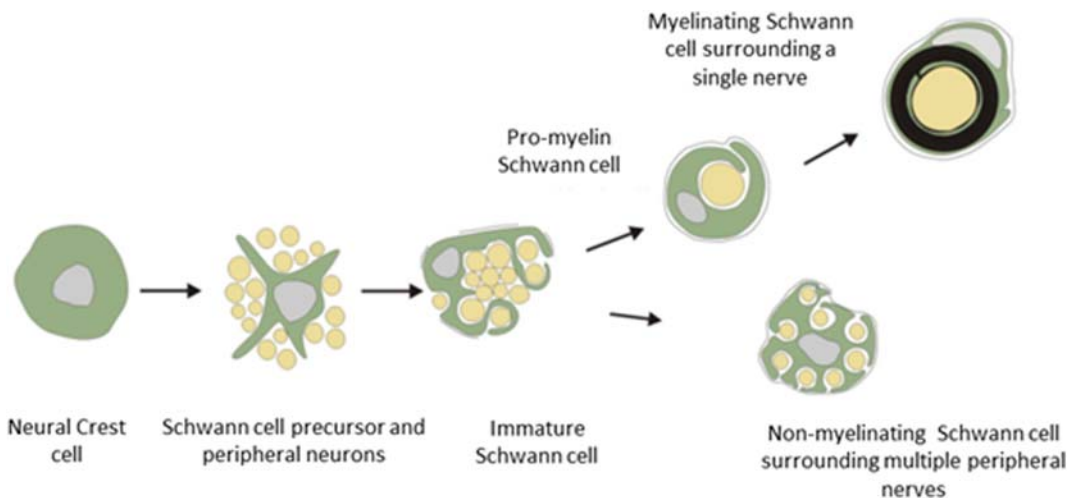


FIGURE 3.12

The subsequent stages in differentiation of NCC into nonmyelinating and myelinating Schwann cells.

that have migrated to the outgrowing axon bundles. The survival and ensheathment of axons by Schwann cell precursors critically depend on the expression and secretion of the neuregulin1 protein by the neurons. Migrating NCCs express the neuregulin1 receptor ErbB3/ErbB2. In turn, the Schwann cells differentiated from the NCC provide **trophic** support and other signals that ensure neuron survival. Without Schwann cells, neurons will die. Vice versa, in the adult but not in the developing fetus Schwann cells will also die when they are deprived from direct axonal contact. Again, this is an example of reciprocal communication between two cell types that are both essential for forming a particular tissue.

3.5.2 Myelinating and nonmyelinating nerve fibers

The fate decision to become either a myelin-forming or a nonmyelin-forming Schwann cell is governed by axonal cues. The exact nature of these cues has long remained elusive and is still not understood in full detail. In myelinating nerve fibers, individual Schwann cells select larger caliber axons in a 1:1 ratio. The extent of myelination, that is, the number of layers of compact myelin and the length of the internode, the distance between two successive nodes of Ranvier, which play an essential role in action potential conduction, correlates with the diameter of the axon. Thus, thicker axons have thicker myelin and longer internodes. In contrast, in nonmyelinating nerve fibers, groups of lower caliber axons remain associated with a single Schwann cell. Nonmyelinating nerve fibers are mostly nociceptive and autonomic neurons. Here, the Schwann cell accommodate and ensheath the individual axons in cytoplasmic extensions of cuffs. These populations of axons-glia are called **Remak fibers**.

3.5.3 Structure of the peripheral nerve sheath

While nerve tracts in the central nervous system are protected by the rigid bony structure of the skull and vertebral column, peripheral nerves lack these protective structures. Instead, they have a compact connective tissue matrix which protects the peripheral nerves. In this structure, three layers can be distinguished, and these have been termed epineurium, perineurium, and endoneurium. The epi- and perineurial cells of the nerve sheath are derived from the mesoderm. The majority of endoneurial cells are derived from the NCC.

While the epineurium is a condensation of connective tissue surrounding the peripheral nerve, the perineurium is a multilayered cellular sheath surrounding individual fascicles of the nerve. The layers consist of concentric sleeves of flattened epithelial cells that interdigitate and are connected by tight junctions at their margins.

The endoneurium mainly consists of a thick layer of collagen fibrils running parallel with the axon—Schwann cell units.

The formation of a normal perineurium largely depends on the Schwann cell—derived signaling molecule desert hedgehog (DHH). DHH is one of three members of the hedgehog family of secreted intercellular signaling molecules that play key roles in embryonic pattern formation and organogenesis. They act as morphogens. Schwann cells are thus not only responsible for survival of the axons, but they are also responsible for orchestrating the differentiation of mesodermal cells into perineurial cells.

3.5.4 Future perspective

The development of the peripheral nerve tissue is orchestrated through a number of cellular and molecular interactions that are now understood in some detail. Schwann cells play a critical role in this process. Next to providing essential support for neurons, Schwann cells orchestrate the development of the protective perineurial sheath. Ideally, artificial nerve grafts that aim to guide and support the regenerating nerve into a distal, denervated nerve stump should include autologous Schwann cells. However, to obtain autologous Schwann cells healthy nerves need to be sacrificed. Much effort is put in place to obtain Schwann cells from various stem cell populations such as embryonic stem cells, iPS cells, stem cells isolated from the hair follicle bulge, and mesenchymal stromal cells. Indeed, various protocols for derivation of Schwann cells from pluripotent stem cell sources are now readily available (Fig. 3.5).

For tissue engineering and/or in situ nerve regeneration in the adult body, the survival of mature Schwann cells in the absence of axonal contact is of physiologic importance as successful regeneration of injured peripheral nerves greatly depends on axonal contact with living Schwann cells in the denervated nerve stump. Indeed, these so-called reactive Schwann cells secrete a range of neurotrophic factors and cytokines, such as nerve growth factor, ciliary neurotrophic factor, leukemia inhibitory factor, and interleukin-6, which stimulate and direct axonal growth. Consequently, successful tissue engineering or even regeneration of peripheral nerves requires the presence of a healthy Schwann cell population which can support and direct nerve outgrowth.

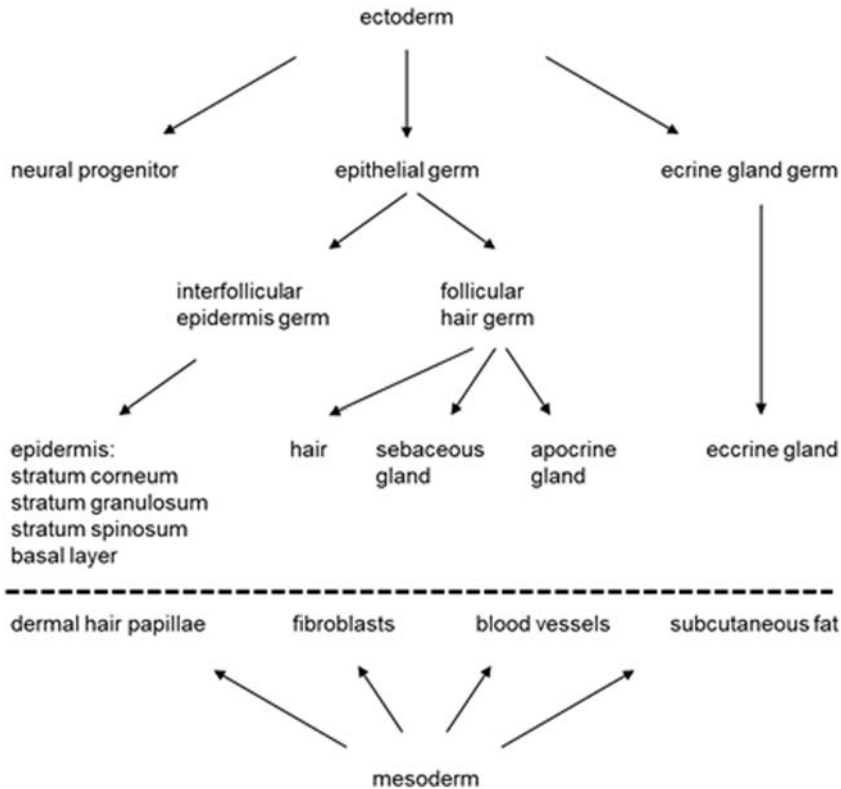
3.6 Embryonic skin development

Tissue-engineered living skin substitutes have found their way into clinical practice for treatment of severe skin defects. While the application of these in vitro generated skin substitutes has greatly improved treatment outcome of severe burn defects, many issues remain to be resolved to end up with an aesthetic and functional equivalent that matches the natural skin. Here the sequential steps and factors involved in embryonic skin development are summarized and correlated with the formation of skin substitutes in vitro. Skin tissue engineering is one of few examples in which developmental engineering has resulted in clinically applicable tissue replacements.

Embryonic skin develops from the ectoderm and mesoderm via a complex process of cell proliferation, differentiation, migration, and apoptosis (Fig. 3.13). The ectoderm is the progenitor of the follicular and interfollicular epidermis and is also the neural progenitor. The mesoderm is the progenitor of the dermis and subcutaneous fat. A timeline for development of human skin structures is given in Table 3.2.

3.6.1 Interfollicular epidermis

In 5-week gestational skin, the epidermis consists of a single layer of ectodermal cells with intermittent areas containing a second suprabasal layer, the periderm (Fig. 3.14). After 7 weeks, Langerhans cells (immune surveillance cells of the skin), derived from bone marrow cells, begin to appear in the developing epidermis. By the 10th week, the stratum intermedium forms between the basal cell layer and the periderm through upward movement of cells from the basal cell layer. The cells of the periderm become

**FIGURE 3.13**

Overview of the origin of cell sources contributing to skin development.

large and protrude into the amniotic cavity. Merkel cells (nerve/touch cells) differentiate from the ectoderm between weeks 8 and 12 of gestation. Around 12 weeks, melanoblasts migrate from the NCC into the ectoderm. These cells later develop into melanocytes—the pigment forming cells in the epidermis. After 21 weeks of gestation, fetal skin becomes thickened due to increasing numbers of stratum intermedium cells and the periderm becomes flattened (Fig. 3.14). At 23 weeks, keratinization takes place in the upper cell layers and small keratohyalin granules form. The cells of the periderm are shed into the amniotic fluid, leaving only fragments of degenerated periderm cells above the keratinized cells of the newly formed stratum corneum. The epidermis continues to form by upward movement of differentiating keratinocytes from the proliferating basal layer until a fully differentiated, stratified epidermis is formed (Fig. 3.14). During the last trimester of gestation (>24 weeks), the newly formed stratum corneum of the fetus is protected from the amniotic fluid by a white, greasy biofilm called vernix caseosa. **Vernix caseosa** consists of water-containing corneocytes embedded in a lipid matrix and the basic structure shows certain similarities with the stratum corneum. After birth, the vernix caseosa is absorbed and the epidermis forms a competent barrier to the environment preventing infection and dehydration of the infant.

Table 3.2 Timelines for human embryonic skin development.

Gestation (weeks)	Fetal length (cm)	Developing structure
5	0.14	Ectoderm: basal single cell layer and intermittent periderm
7		Langerhans cell precursors from hematopoietic origin
8		Mesoderm: loosely arranged mesenchymal cells embedded in ground substance; merkel cell differentiation from ectoderm
10	5.0	Epithelial germ: basal cell layer, stratum intermedium, periderm
12		Epithelial germ: melanocyte precursors in intermediate layer; early dermis: argyrophilic reticulum fibers (collagen III), abundant fibroblasts
13		Eccrine glands start to develop
17	17	Hair and sebaceous gland development
19		Epidermis: basal layer, 2–3 intermediate layers, flattened periderm
20		Apocrine glands;
22	19	Fat cells mainly in brown fat (very little white fat)
23		Dermis: elastic fibers
24		Epidermis: keratinization in stratum intermedium (keratohyalin granules), premature stratum corneum
32	40–50	Formation of vernix caseosa
36		Papillary and reticular dermis
		Fully developed immune competent skin



FIGURE 3.14

The subsequent steps in human skin development from 5 weeks of gestation to postnatal skin.

3.6.2 Follicular epidermis

The ectoderm basal cell layer proliferates and differentiates into the keratinizing interfollicular epidermis as described above. Additionally, the ectoderm forms the eccrine glands (sweat) and hair

germs which then further differentiate into hair, sebaceous glands, and apocrine glands (Fig. 3.14). In early gestation (13 weeks), when fetal skin is composed of two or three epidermal layers, eccrine glands begin to form. Eccrine gland islands composed of epidermal cells gradually migrate down into the dermis to form the juvenile sweat glands at weeks 18–20. Hair follicles begin to form at 17 weeks. The formation of these follicles represents a prototypic interaction between the neuroectoderm and mesoderm that is provided by three different stem cell sources: epidermal, neural crest, and mesenchyme. The epidermal keratinocyte mass that differentiates into hair follicles and sebocytes buds into the deeper layers of the dermis (Fig. 3.14). Beneath each bud lies a group of mesenchymal cells (fibroblasts) from which the dermal hair papillae and connective tissue sheath are later formed. NCCs give rise to the melanocytes of the hair follicle pigmentary unit. After 21 weeks of gestation, the structures of skin appendages (eccrine glands, hair follicles, and sebaceous glands) can be detected (Fig. 3.14). After 28 weeks of gestation, as fetal skin becomes even thicker, the number of eccrine glands and hair follicles is increased, and the structure of the appendages becomes mature. At birth, the sebaceous glands are considerably larger than they are in infancy and secrete the vernix caseosa.

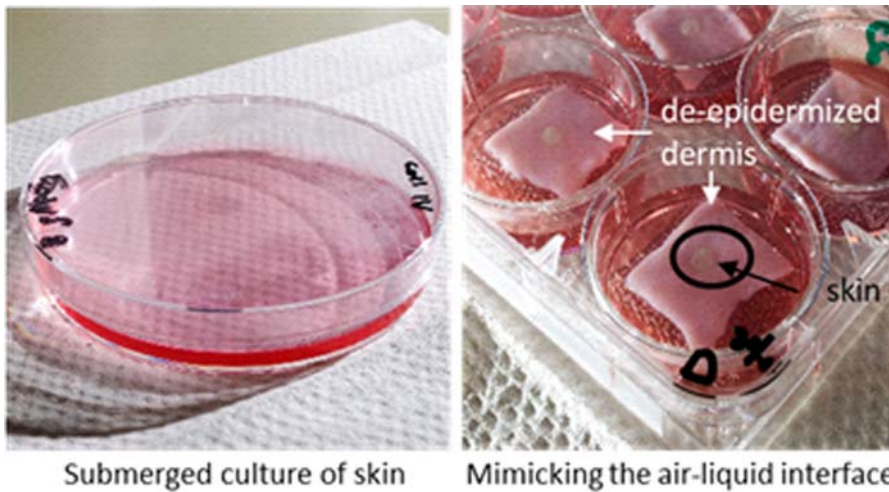
3.6.3 Dermis

In 5-week gestational skin, the dermis consists of loosely arranged mesenchymal cells that are embedded in a ground substance (Fig. 3.14). At 12 weeks, argyrophilic reticulum fibers appear. As these fibers increase in number and thickness, they arrange themselves in bundles. Simultaneously, mesenchymal cells develop into fibroblasts and endothelial cells. The fetal dermis shows many more fibroblasts than adult dermis (compare middle and right panel Fig. 3.14). Also, the dermis of the fetus contains a large amount of the extracellular matrix protein collagen type III, in contrast to adult skin which contains a large amount of collagen type I. Elastic fibers appear in the dermis at 22 weeks. As gestation continues, elastic fibers increase in number until at 32 weeks, a well-developed network is formed in the reticular and papillary dermis which is indistinguishable from that found in newborn infants.

3.6.4 Tissue engineering of embryonic and newborn skin

The various stages of skin development can be mimicked *in vitro* by adjusting and finely tuning the culture conditions under which the cells are grown. One should not just consider the tissue itself (in this case skin) but also the environment around the tissue. For example, embryonic ectoderm is in a wet environment engulfed by amniotic fluid on one side and the developing mesoderm rich in fibroblasts on the other side. This provides an extremely wet and nutrient-rich environment. Upon birth, the environment changes significantly. The skin is now exposed to the air. Nutrients reach the epidermis by diffusion from blood vessels in the underlying dermis. Therefore, conditions switch from a wet to a dry environment and also from a nutrient-rich to a nutrient-poor environment. This transition is thought to trigger the final stage in the formation of fully developed skin, thus forming a competent barrier to the environment and can be mimicked *in vitro* when engineering skin by changing Petri dish cultures from a submerged condition to an air exposed condition (Fig. 3.15).

Cultures that resemble early ectoderm can be generated from newborn or adult keratinocytes by culturing the keratinocytes on a dense feeder layer of lethally irradiated fibroblasts completely submerged in culture medium (Fig. 3.16, compare left and right). The fibroblasts provide a contact point

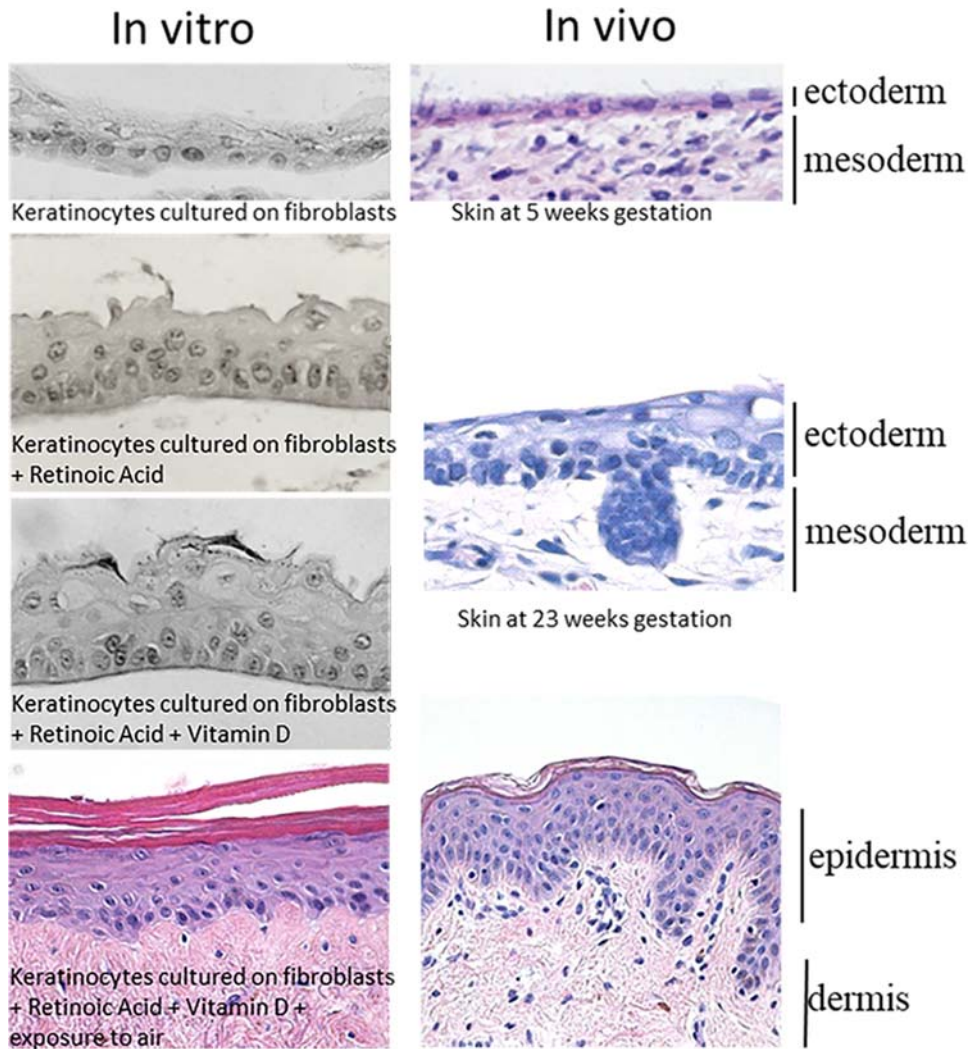
**FIGURE 3.15**

A Petri dish showing experimental set up for culturing skin cells on top a square piece of deepidermized dermis under submerged (*left*) or at the air—liquid interface (*right*) conditions mimicking the environmental change which occurs around birth.

for the initially seeded keratinocytes to nestle up to, but importantly the fibroblasts are metabolically active resulting in the secretion of growth factors and cytokines essential for keratinocyte viability and proliferation. Again, this is an example of the importance of cross-talk between, in this case, epidermal cells and the underlying mesenchyme which directs tissue formation and maturation. The result is a keratinocyte sheet which consists of approximately two layers of unkeratinized epidermis hardly showing any similarity to newborn skin. The keratinocyte sheet cultured in this way visibly resembles early ectoderm at 5 weeks gestation (Fig. 3.16).

In order to progress to the next stage of embryonic epidermal development *in vitro*, additional nutrients are required. Addition of, for example, retinoic acid (a factor known to be involved in healthy skin development) to the primitive ectoderm-like culture stimulates cell division and upward migration of keratinocytes. This increases the number of cell layers and results in cultures which visibly resemble embryonic skin at 21 weeks gestation (Fig. 3.16). Addition of vitamin D in combination with retinoic acid further increases the number of layers, epidermal differentiation, keratinization, and the appearance of small keratohyalin-like granules in the upper layers (Fig. 3.16). These cultures visibly resemble embryonic skin at approximately 23 weeks gestation.

In order to mimic the final stage in epidermal development, the culture environment has to be changed significantly copying the environmental change at birth. The most profound change is exposure of the skin to the air in place of the warm and wet environment of the amniotic fluid. *In vitro*, this can be mimicked by raising the keratinocyte cultures to the air—liquid interface and culturing on a scaffold or matrix, e.g., human dermis (Fig. 3.16). The transition from submerged culture conditions to air exposed culture conditions stimulates the final step in skin development—the formation of the stratum corneum.

**FIGURE 3.16**

The development of fetal skin can be mimicked in vitro by coculture of keratinocytes with lethally irradiated fibroblasts embedded in the dermis, medium additives and air exposure.

The developing dermis can be copied in vitro by coculturing fibroblasts and endothelial cells. Cell–cell interactions and optimal culture conditions stimulate individual endothelial cells to migrate toward each other to form capillary-like tubes. Fibroblasts synthesize extracellular matrix components thus forming a human collagen–elastin rich dermal matrix similar to in vivo.

3.6.5 Cell–cell interactions and growth factors

The skin development and thereafter the maintenance of skin integrity (homeostasis) is dependent on a complex interplay between cell types within the ectoderm (epidermis) and mesoderm (dermis). **Cell homing** to the correct location, proliferation, and differentiation is regulated by growth factors, cytokines, and chemokines that act in **autocrine** and **paracrine** loops. The result is the formation of a fully developed skin consisting of keratinocytes and fibroblasts with constant ratios of keratinocytes: melanocytes (36:1) and keratinocytes: Langerhans cells (53:1). The formation of cell units can be mimicked in vitro and gives an insight into embryonic skin development. Melanocytes cocultured with keratinocytes under submerged conditions (ectoderm-like culture) maintain a constant ratio for up to three passages (3 weeks) and when coseeded onto acellular dermis and cultured at the air–liquid interface form a differentiated epidermis interdispersed with melanocytes in the basal layer. Melanosomes formed in the melanocytes are transferred to keratinocytes and cap the nuclei just as in vivo in order to protect the dividing keratinocyte population from harmful ultraviolet irradiation after birth. It has been reported that fetal keratinocytes possess a greater stimulatory effect on proliferation of melanocytes than neonatal keratinocytes, as fetal keratinocytes produce and release more mitogens than newborn keratinocytes. Similar to melanocytes, Langerhans cell precursors can be introduced into submerged keratinocyte cultures which when air-exposed develop into fully immunocompetent Langerhans cells in the epidermis in ratios similar to those found in fully developed skin. Cocultures of keratinocytes and fibroblasts (irradiated feeder layer) maintain the keratinocyte stem cell population in vitro and enable large amounts of keratinocytes, including stem cells, to be amplified for transplantation as sheets onto large burns wounds. Upon transplantation, the ectoderm-like culture develops into a differentiated adult epidermis as the environmental conditions change (submerged, nutrient-rich environment changes to air-exposed, nutrient-poor environment). Soluble factors secreted by keratinocyte–fibroblast interactions are involved in the formation of the basement membrane. The **basement membrane** is essential for the attachment of the epidermis to the dermis and heritable defects can result in blistering (bullous disease). Soluble factors secreted by keratinocytes result in homing of fibroblasts into the dermis, whereas soluble factors secreted by fibroblasts stimulate formation of the epidermal layers (basal layer, spinous layer, granular layer, and stratum corneum). One of these factors is keratinocyte growth factor (KGF or FGF7) which is a member of the FGF family of growth factors (Fig. 3.17). Supplementation of FGF7 to the culture medium induces keratinocyte proliferation and differentiation and can be used to replace living fibroblasts in the dermal matrix (Fig. 3.17). Table 3.3 summarizes the properties of some keratinocyte- and fibroblast-derived growth factors involved in skin development.

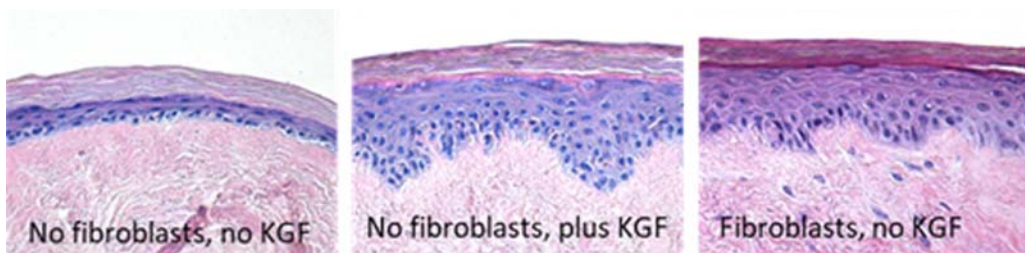


FIGURE 3.17

KGF (FGF7) can replace lethally irradiated fibroblasts stimulating keratinocyte proliferation.

Table 3.3 Some of the growth factors secreted by keratinocytes and fibroblasts which are involved in skin development.

Growth factor	Secreted by	Target cell	Property
Bone morphogenic protein	?	Ecoderm/KC	Differentiation of ectoderm and epidermis
Noggin	Mesoderm	Ectoderm	Neural differentiation, hair follicle formation
Epiregulin	KC	KC	Suppression of ectoderm, Proliferation
Epidermal growth factor	Hair follicle?	KC/Fib	Proliferation, migration
Keratinocyte growth factor	Fib	KC	Proliferation, differentiation
Tumor growth factor- α	KC/Fib	KC/Fib	Differentiation, migration, extracellular matrix synthesis
Vascular endothelial growth factor	KC	Endo	Angiogenesis
Tumor necrosis factor- α	KC/Fib	KC/Fib/LCp	KC/Fib proliferation, LCp differentiation to LC
Tumor growth factor- β	KC	Fib/LCp	Fibroblast differentiation to myofibroblast, LCp differentiation to LC
Granulocyte macrophage-colony stimulating factor	KC	LCp/MC	LCp differentiation to LC, MC proliferation, melanogenesis, dendritogenesis
Basic fibroblast growth factor	KC	MC/Endo/Fib	Proliferation, angiogenesis
Hepatocyte growth factor	KC/Fib	MC	Proliferation
α -MSH	KC	MC	Melanogenesis, dendritogenesis
Nerve growth factor	KC	MC	Melanogenesis, dendritogenesis
Endothelin 1	KC	MC	Proliferation, melanogenesis, dendritogenesis
Stem cell factor	KC/Fib	MC	Proliferation, melanogenesis, dendritogenesis

Endo, *endothelial cell*; Fib, *fibroblast*; KC, *keratinocyte*; LC, *Langerhans cell*; LCp, *Langerhans cell precursor*; MC, *melanocyte*.

3.6.6 Future perspective

The development of embryonic skin involves environmental, matrix, and cell–cell interactions. Inspired by developmental biology, these processes can be mimicked in vitro to engineer skin substitutes. Very important in this engineering process is the fine-tuning of differential and sequential secretion of soluble factors which regulate cell growth and differentiation. In vitro, a number of phases of embryonic skin development can be mimicked, such as development of the interfollicular epidermis including keratinocytes, melanocytes, and Langerhans cells. Also, a capillary network in a fibroblast-populated dermal matrix can be generated from a mesenchymal cell mix. However, the formation of appendages such as hair follicles and eccrine glands, which involves extensive dermal and epidermal interactions, is still challenging.

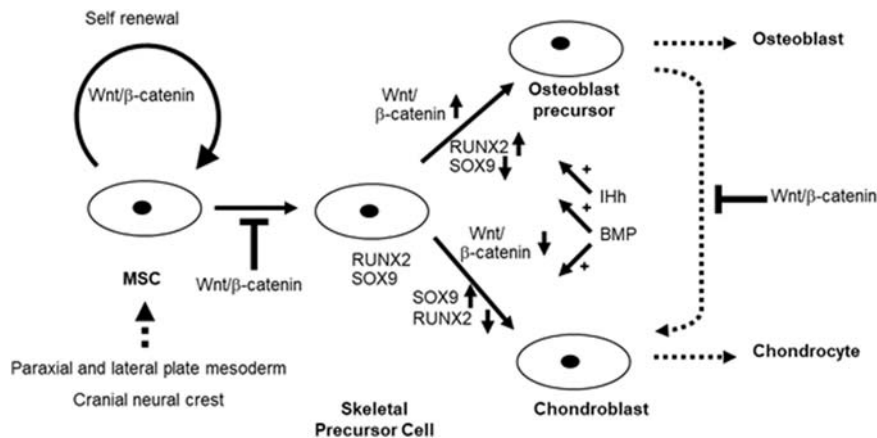
3.7 Bone development

Major challenges for tissue engineering of the skeleton are (i) the repair of damaged articular cartilage covering the distal ends of the bone in the joints; (ii) the repair of nonunion bone fractures, and (iii) the improvement of the union of synthetic prostheses with native bone (see [Chapter 16 Cartilage and bone regeneration](#)). The purpose of this section is to provide a general overview of skeletogenesis during fetal development including the origin of the bone forming and degrading cells and the basic principles underlying the maintenance of skeletal integrity during adulthood.

The skeleton is a dynamic living tissue largely consisting of an **extracellular matrix** combined with matrix forming and degrading cells. The extracellular matrix can be divided into two types, bone and cartilage, which differ in their matrix composition and physical properties in line with their respective functions. Three cell types are involved in skeletal formation: the cartilage producing **chondrocyte**, the bone forming **osteoblast**, and the cartilage and bone resorbing **osteoclast**. Three cell lineages are responsible for the formation of the skeleton during embryonic development. The mesodermal paraxial and lateral plate mesoderm and the neuroectoderm-derived cranial NCC. The lateral plate mesoderm will form the long bones in the limbs. The paraxial mesoderm will form the axial skeleton (vertebral column and rib cage) and together with the NCC will shape the craniofacial bones. The formation of bones starts during the fourth week of human development and begins with the formation of the bones at the base of the skull. New bones are formed in an **anterior–posterior** direction. With exception of the bones in the jaws, the formation of the craniofacial bones begins later. The shape of each bone is specifically adapted to its function, which depends on its place in the skeleton. Even before the onset of skeletogenesis, cells at the site of the future bone already contain information on the shape and structure of the bone they will form.

3.7.1 Skeletal precursor cells

Osteoblasts and chondrocytes are both derived from the multipotent mesenchymal stromal cells or also often referred to as MSCs (see [Chapter 2 Stem cells](#)). These cells remain present in the bone from onset of bone formation onwards into adult life. Here these cells play an important role in the continuous cycle of bone formation and degradation by providing a source for new osteoblasts. Signaling by the WNT family of morphogens plays an important role in the maintenance of the undifferentiated stem cell population. WNTs stimulate stem cell proliferation and renewal and simultaneously block

**FIGURE 3.18**

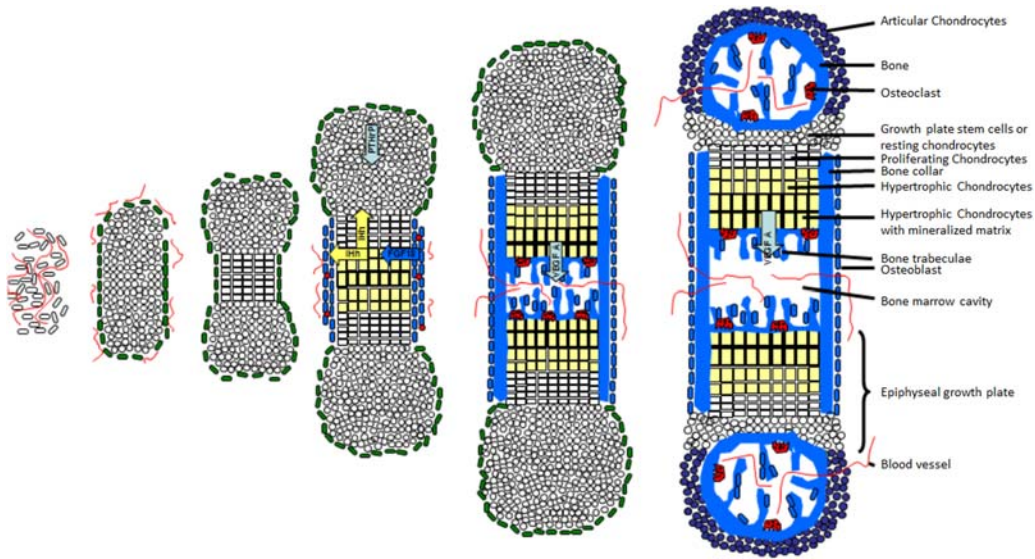
The role of WNT signaling and the transcription factors RUNX2 and SOX9 in differentiation of bone forming osteoblasts and cartilage forming chondrocytes.

the initiation of differentiation by increasing the nuclear localization and the transcription potential of the transcription factor β -catenin, an essential and critical intracellular mediator of the WNT signal transduction pathway.

At sites of future bones, the MSC will give rise to a skeletal precursor cell which can differentiate into either an osteoblast or a chondrocyte. The direction of differentiation depends on the activity of two transcription factors: SOX9 and RUNX2. SOX9 is indispensable for chondrocyte differentiation, while RUNX2 is indispensable for osteoblast differentiation. The skeletal precursor cell expresses both transcription factors. Activation of the WNT signaling pathway inhibits SOX9 and potentiates RUNX2 activity resulting in osteoblast differentiation. In contrast, inhibition of WNT signaling activates SOX9 activity which stimulates the formation of chondrocytes (Fig. 3.18).

3.7.2 Endochondral ossification

The axial and appendicular skeleton is formed by **endochondral ossification**, i.e., the skeletal elements are preshaped in a cartilaginous mold which is subsequently replaced by bone. This process starts with the condensation of loosely connected mesenchymal cells at sites of the future bones (Fig. 3.19). This is accompanied with a rearrangement of the vasculature resulting in an avascular condensed mesenchyme surrounded by blood vessels. The combination of increased cell–cell contacts, low oxygen conditions due to the absence of vessels, and low levels of WNT signaling is believed to trigger the initiation of chondrocyte differentiation. TGF β and other members of the BMP superfamily play a critical role in this initial phase of chondrocyte differentiation. This phase is characterized by the production of an abundant extracellular matrix consisting predominantly of collagen type 2a1 and glycosaminoglycans, which are typical cartilage markers. In the middle of the cartilage anlage, chondrocytes start to arrange in columns and display high proliferative activity. Somewhat later these chondrocytes stop proliferating and differentiate further into hypertrophic chondrocytes. This is characterized by a dramatic increase of

**FIGURE 3.19**

The subsequent stages in endochondral ossification in which long bones are preshaped in a cartilaginous mold.

the cell's volume and a reshuffling of the extracellular matrix, which now contains collagen type 10 instead of collagen type 2.

The hypertrophic chondrocytes start to mineralize their matrix forming the **primary ossification center** and subsequently die by apoptosis. At the same time, the chondrocytes signal to the surrounding perichondria cells in which osteoblast precursors reside by producing Indian hedgehog (IHH). Like Desert hedgehog (DHH), IHH belongs to the hedgehog family of morphogens. IHH initiates osteoblast differentiation and the formation of the bone collar. It is the most important coupling factor between chondrocyte and osteoblast differentiation during endochondral ossification. Vice versa, the osteoblasts in the bone collar produce FGF18 which regulates chondrocyte proliferation. IHH also signals to the periarticular region of the growing long bones where it stimulates the expression of parathyroid hormone–related peptide which critically regulates the pace of chondrocyte differentiation by inhibiting hypertrophic differentiation. The hypoxic conditions in the center of the developing long bone induce the expression of the growth factor VEGF by mineralized hypertrophic chondrocytes. VEGF recruits blood vessel ingrowth from the lateral perichondrium. With the in growing vessels, both osteoclasts and osteoblasts enter the bone anlage resulting in the resorption of cartilage, replacement by newly formed bone, and the formation of the bone marrow cavity. In this process, a cartilaginous matrix containing collagen type 10 and glycosaminoglycans are replaced by a mineralized bone matrix predominantly consisting of collagen type 1. Somewhat later, this process repeats itself in the cartilaginous heads of the growing long bones resulting in the formation of the **secondary ossification center**. The secondary ossification center demarcates the separation of two types of chondrocytes: **articular chondrocytes** that cover the distal ends of the bones in the articulating joints and epiphyseal growth plate chondrocytes that are responsible for bone elongation. Experimental evidence suggests that

specification of the articular versus the growth plate chondrocyte occurs already at the first stages of endochondral ossification.

3.7.3 Intramembranous ossification and osteoblast differentiation

The flat bones of the skull and the flat part of the clavicle are formed by **intramembranous ossification**. In this process, cells directly deposit a mineralized bone matrix without a cartilage intermediate. This process also starts with condensation of mesenchymal cells. In contrast to endochondral bone, these condensations occur in vascularized regions of mesenchyme. The condensed cells directly differentiate into osteoblasts which start to produce a mineralized bone matrix. The sequential activation of two transcription factors is essential for the formation of osteoblasts from skeletal precursor cells. This differentiation route is initiated by RUNX2 and requires relatively high levels of WNT signaling. The subsequent activation of a second transcription factor, Osterix, is needed for further maturation of the cells and the production of a mineralized bone matrix.

The life cycle of the osteoblast can be divided into various phases. In the first phase, the number of osteoprogenitors is increased by rapid proliferation. Subsequently, the cells begin to secrete large quantities of extracellular matrix predominantly consisting of collagen 1 followed by a maturation phase in which the matrix is prepared for matrix mineralization. Each of these phases is characterized by the expression of typical markers (Fig. 3.20). At the end of the life span, the osteoblast has three choices: it can die by apoptosis, it can become fully embedded in the extracellular bone matrix and differentiate further into an osteocyte, or it can become a quiescent bone lining cell that covers the bone surface. Osteocytes are single cells fully surrounded by a mineralized bone matrix. They are in close contact with each other and the bone surface with a network of cell extensions. These cell protrusions are

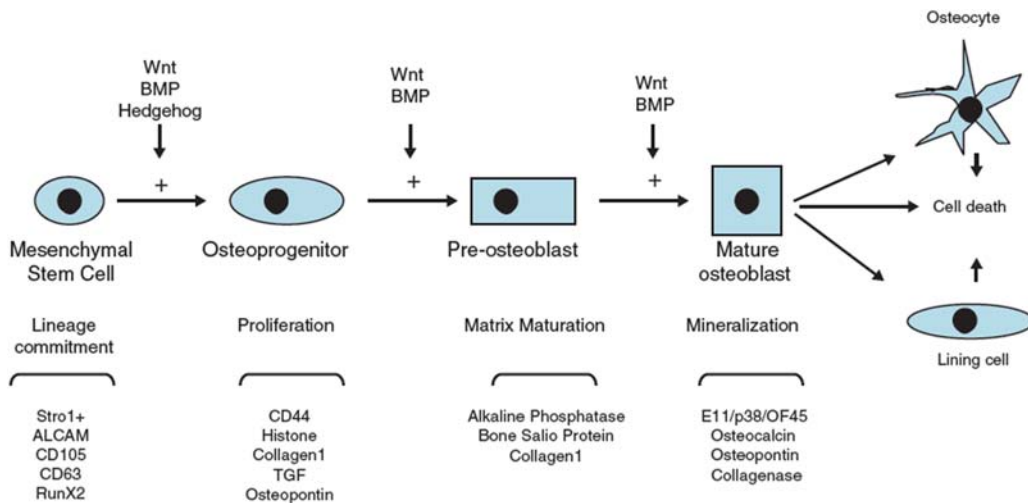


FIGURE 3.20

The multistep differentiation of mesenchymal stem cells into osteoblasts and frequently used markers to distinguish the subsequent stages in differentiation.

localized in small channels, the **canaliculi**. The lining cells are quiescent cells which are no longer involved in bone formation. Upon the appropriate signals, these cells can, however, resume their activity and start participating again in the formation of a bone matrix.

A large number of growth factors are involved in the regulation of osteoblast differentiation. Members of the TGF β /BMP superfamily, particularly the BMPs, IHH, and WNT family members, can mediate initiation of osteoblast differentiation from uncommitted precursors. These factors act, among others, by inducing the expression of RUNX2. Other factors like insulin-like growth factor I, TGF β , and FGFs to name a few play a role in osteoblast proliferation, matrix production, and mineralization. Of particular interest are members of the WNT family, which are involved in successive stages of osteoblast differentiation such as in the initiation of osteoblast differentiation, matrix production, and osteoblastic cell death. Furthermore, they are involved in the regulation of the coupling between bone formation and bone resorption. Besides control by locally produced growth factors, bone formation by osteoblasts is controlled by **systemic factors**, such as sex steroids, growth hormone and parathyroid hormone, and by the hypothalamus via the sympathetic nervous system.

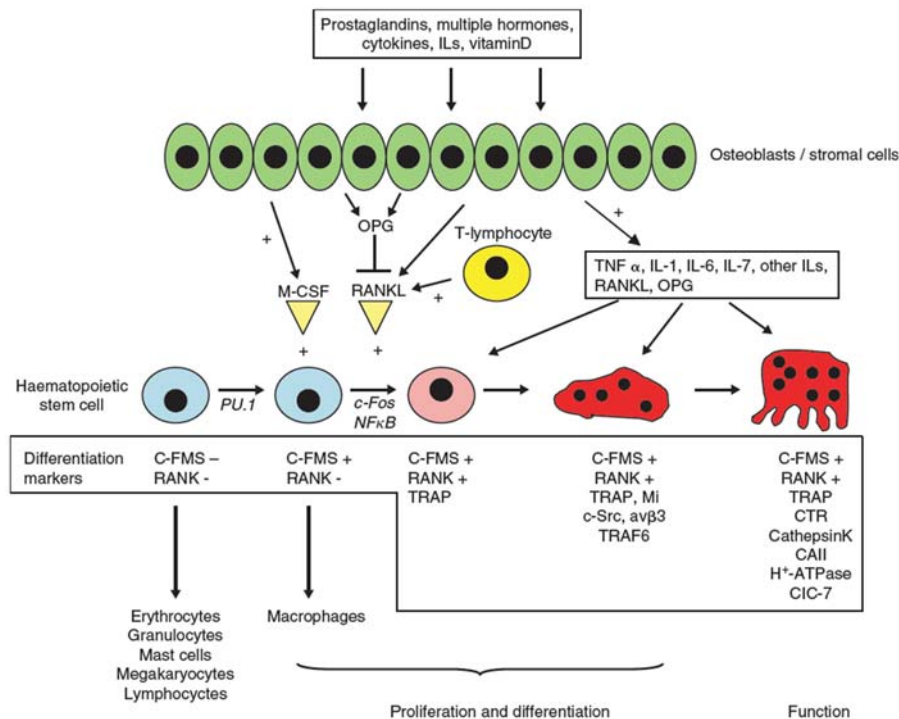
3.7.4 Osteoclast differentiation

The osteoclast is a multinucleated highly specialized cell specifically equipped for the resorption of a mineralized matrix (Fig. 3.21). Osteoclasts are derived from the hematopoietic stem cell. This cell gives rise to erythrocytes, granulocytes, mast cells, megakaryocytes, lymphocytes, and macrophages. The derivation of osteoclasts from this lineage requires a direct interaction between the osteoclast precursor and the osteoblast involving the expression of receptor activator of nuclear factor kappa β (RANK) by the osteoclast precursor and the membrane-associated ligand (RANKL) by the osteoblast. The interaction between RANK and RANKL is required for all subsequent stages of osteoclastogenesis including the fusion of mononucleated precursor cells into a multinucleated functional osteoclast. The subsequent stages of osteoclast development are characterized by specific marker genes (Fig. 3.21). Various cytokines expressed by, among others, osteoblasts stimulate osteoclast maturation and bone resorption.

The formation of osteoclasts is tightly linked with osteoblast differentiation, since the expression of RANKL is controlled by the osteoblast specific transcription factor RUNX2.

3.7.5 Tissue engineering of bone

MSCs derived from adult bone marrow are the most frequently used cell source to engineer bone. In most strategies, their capacity to differentiate into bone forming osteoblasts in a process that resembles intramembranous ossification is exploited. In the treatment of critical size bone defects, limited clinical successes have been achieved. Based on lessons from bone formation in the embryo, this could have been predicted. Bone fractures normally heal, with a few exceptions, in a process resembling endochondral ossification. Recapitulation of this process in a tissue engineering strategy to treat large bone defects implies that one should not aim at directly making bone out of MSCs, but one should first make a cartilage anlage. After implantation, this cartilage will be replaced by bone through endochondral ossification which is organized by the tissue itself through interaction with host tissue. Implantation of tissue-engineered cartilage instead of bone has various advantages. For example, cartilage is an avascular tissue and chondrocytes are resilient to low oxygen concentrations. In contrast, bone is highly

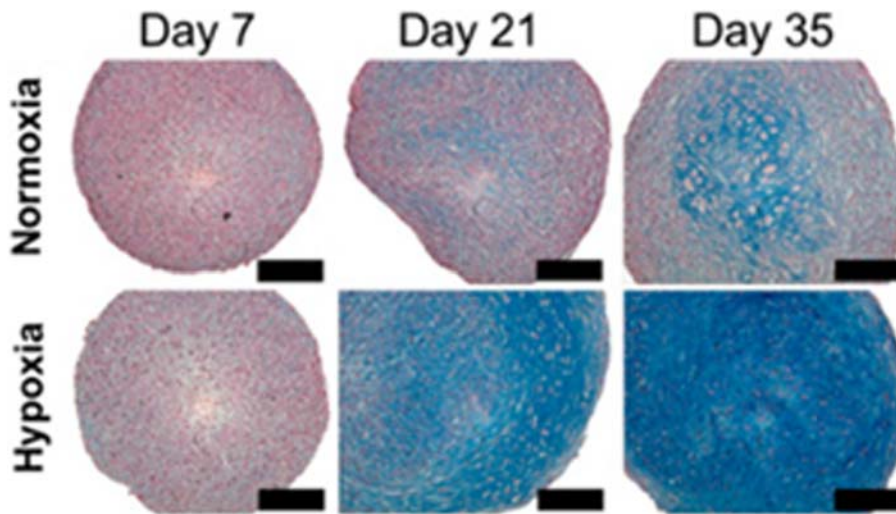
**FIGURE 3.21**

Osteoclasts are derived from hematopoietic stem cells in a multistep differentiation process critically depending on the interaction with osteoblasts.

vascularized and metabolically very active. Lack of oxygen as a consequence of the absence of a vasculature in many tissue-engineered constructs is the main reason for transplant failure. In endochondral ossification, the cartilage self-organizes the ingrowth of blood vessels, which is the first step leading to cartilage degradation, and its replacement by bone. This is an example how tissue engineers have started to exploit the insights from developmental biology into an engineering strategy using the modularity and self-organizing principles of developing tissue to obtain a robust strategy for treatment of bone defects.

3.7.6 Tissue engineering of articular cartilage

Most protocols for the derivation of cartilage from MSC sources mimic the developmental path of cartilage formation in the embryo. It starts with condensation of stem cells in pellets using nutrient poor culture medium in the presence of the growth factor TFG β that is also responsible for driving chondrogenesis in the embryo. These pellet cultures mimic the condensation of mesenchymal cells in the cartilage anlage. Furthermore, cartilage tissue is exposed to hypoxic conditions due to the lack of blood vessels. This environmental aspect can be mimicked by culturing the cell pellets under low oxygen conditions. Indeed, low oxygen conditions dramatically stimulate cartilage formation in vitro (Fig. 3.22).

**FIGURE 3.22**

Culturing cell pellets of mesenchymal stem cells in the presence of TGF β under low oxygen conditions stimulates the deposition of a glycosaminoglycan rich extracellular matrix which is stained blue.

3.7.7 Future perspective

Over the past years, the tissue engineering field is gradually changing. While at its infancy, tissue engineering was mainly based on trial and error, this new discipline is rapidly developing into a technology-based discipline comparable to other branches of engineering. Reiteration of processes in developmental biology is now exploited to build in vitro tissues. Concepts such as modularity, robustness, and self-organization, which are at the basis of the formation of organs, will be more and more incorporated in tissue engineering strategies, while novel technologies such as bioprinting of living tissues will be used to engineer more complex geometries and/or to integrate blood vessels in tissue engineered constructs. This trend will continue in the coming years resulting in maturation of a new discipline in the formation of tissues called developmental engineering.

Summary

- Recapitulation of developmental processes in combination with typical engineering disciplines is known as developmental (re)engineering and forms the basis of most, if not all, tissue engineering procedures.
- Tissue formation depends on the timely and subsequent interaction between multiple stem or progenitor cells derived from various origins. Cell migration, cell–cell contact, auto- and paracrine signaling, and also environmental cues like pulsatile flow or exposure to air are some of the processes that are used for the specification of cells into a functionalized tissue.
- In gastrulation, the three germ layers ectoderm, mesoderm, and endoderm are formed by a complex process involving cell–cell interactions, cell movements, and gradients of morphogens by which the body plan is established.

Continued

Summary—continued

- Pluripotent NCCs originate from the neural plate border. They migrate via clearly defined patterns to all tissues in the body where they actively participate in tissue formation. They give rise to cell types of ectodermal, mesodermal, and endodermal origin.
- The formation of the heart is a complex and time-consuming process. Mesoderm-derived cells and NCC play a pivotal role in the formation of the embryonic heart. Initially the heart is formed as a single beating tube that by a series of geometrical changes transforms into a four-chambered septate beating heart.
- In blood vessel formation, endothelial cells are derived from the mesodermal angioblast. The SMCs and pericytes are derived from the mesoderm or the neural crest. Pulsatile blood flow and communication between endothelial cells and surrounding SMCs or pericytes will shape and define the identity of the vessel into either arteria or veins. In the absence of blood flow, vessels will not develop properly or even deteriorate.
- In peripheral nerve tissue, the neurons and glial cells (also called Schwann cells) are derived from either the neuroectoderm or neural crest, while the nerve sheath is derived from the mesoderm. Neurons can only survive in close contact with Schwann cells. Vice versa, mature Schwann cells can only survive in close contact with neurons. Only immature Schwann cells can survive without axonal contact.
- In adult skin, the follicular and interfollicular epidermis is derived from the ectoderm. The mesoderm is the progenitor of the dermis and subcutaneous fat. The development of embryonic skin involves environmental, matrix, and cell—cell interactions. These processes can be mimicked to some extent in vitro by changing the culture conditions from a wet to air-exposed environment and by coculture of dermal fibroblasts and keratinocytes.
- The major bone forming cells, the osteoblast and chondrocyte, are derived from the paraxial and lateral plate mesoderm and the neuroectoderm-derived cranial neural crest. The bone-degrading osteoclasts are derived from the hematopoietic stem cell. Bone is formed either by intramembranous ossification or by endochondral ossification. In intramembranous ossification, MSCs directly differentiate into a bone matrix depositing osteoblast. In endochondral bone formation, skeletal elements are performed in a cartilaginous mold that is replaced by bone.
- The differentiation paths and the molecular mechanisms by which pluripotent cells in the embryo differentiate into tissue specific cell types have largely been resolved and these processes can be mimicked in the laboratory. It is now possible to derive almost all cell types in the human body using dedicated stepwise differentiation protocols that recapitulate key steps that take place in the embryo.

Classical experiment

Isolation and culture of the first pluripotent stem cells

Already in the “50s of the 20th century, it was recognized that the 129Sv strain of mice exhibits a high incidence in the formation of spontaneous testicular tumors, the so-called teratocarcinomas. Teratocarcinomas or teratomas can also be induced in mice by the grafting of early embryos into

extrauterine sites. Individual cells from these tumors could be transplanted to other mice where they formed new tumors, demonstrating the existence of cells with self-renewal capacity or stem cells in the tumors. Teratocarcinomas contain a wide variety of differentiated cells that are derivatives of the stem cells. The exact potency of these stem cells remained unclear until chimeric mice were

Classical experiment—continued

created by injecting few of the tumor cells into blastocyst stage embryos. The tumor cells started to participate in the development of many cells and tissues, including germ cells. This demonstrated that the teratocarcinoma cells were pluripotent. The cell lines that were derived from teratocarcinomas were called embryonal carcinoma cell lines. The pluripotency of the embryonal carcinoma cells raised many questions. These cells were after all derived from embryos. It was hypothesized that the early embryo contained cells that divided and remained pluripotent unless they received signals for differentiation, as normally occurs during embryogenesis. It seemed logical to assume that these cells could be directly isolated from the embryo and kept in culture.

Martin Evans and Matthew Kaufman, then from the University of Cambridge, were the first to directly generate progressively growing cultures from preimplantation mouse embryos. By delaying the implantation of blastocyst stage embryos, they obtained embryos with a bigger-than-normal inner cell mass. Using their knowledge of culture media obtained from working with embryonal carcinoma cells, they were able to culture cells from these large blastocysts. The cells strongly resembled embryonal carcinoma cells; they too formed teratomas when injected

into mice and differentiated in vitro. Evans and Kaufman published their seminal paper on the derivation of pluripotent embryonic cells in 1981. In the same year, Gail Martin from the University of California independently established pluripotent cell lines from mouse blastocysts. Instead of using delayed implantation embryos, Martin was able to successfully culture the embryonic cells by using a feeder layer of fibroblasts and conditioned medium from embryonal carcinoma cells. Martin coined the term embryonic stem (ES) cells for these embryo-derived cell lines. The pluripotency of the ES cells was convincingly demonstrated by the formation of healthy germ-line chimeras.

In the four decades following this seminal discovery, insights from developmental biology during organogenesis have led to the establishment of step-wise protocols for the differentiation of nearly all cell types starting from ES cells in laboratory conditions. These protocols are based on recapitulating subsequent critical steps in the developmental path from pluripotent stem cells in the embryo into tissue-specific cells.

Evans MJ, Kaufman MH. Establishment on culture of pluripotential cells from mouse embryos. Nature. 1981;292:154–156.

State-of-the-art experiment

Realizing that most bones in our body are formed and healed by endochondral ossification, Martin and coworkers started to explore whether this developmental biological phenomenon could be used to generate bone. They started with human mesenchymal stromal cells. While others focused on directly making bone via intramembranous ossification, Martin started to make cartilage first. The cartilage templates were subsequently implanted ectopically in a mouse. Indeed, the cartilage templates were efficiently replaced by functional bone containing a mineralized matrix. The efficacy depended on the maturation state of the cartilage. Efficient replacement by bone was shown only in cartilage constructs containing hypertrophic cartilage. The ectopic bone was vascularized and

contained a bone marrow compartment in which hematopoiesis took place. Hypertrophic cartilage was capable to recruit the ingrowth of blood vessels from the host tissue. In addition, the cartilage was able to recruit osteoblast precursors from the host which started to deposit bone. Cells from the bone marrow compartment, such as the osteoclasts, were also derived from the host. Some human MSCs also differentiated into osteoblasts particularly at the pericortical region. The bone in this region was thus derived from both implanted human cells and host cells. Importantly, the underlying morphogenetic process was structurally and molecularly similar to the temporal and spatial progression of long bone development in the limbs. The study by Martin and coworkers provides a model for

Continued

State-of-the-art experiment—continued

fundamental and translational studies on bone morphogenesis and regeneration by invoking a developmental engineering paradigm.

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3.9 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
 1. What is the name of the process during embryogenesis in which the three germ layers are established?
 2. Name the three germ layers and indicate for each of these germ layers organs that are derived from these cells.
 3. When do embryonic cells of the inner cell mass of the blastocyst lose pluripotency?
 4. What is a morphogen?
 5. Name a few examples of growth factors that can act as morphogens.
 6. At which position in the embryo do neural crest cells arise?
 7. Why is it difficult to isolate neural crest cells for tissue engineering purposes?
 8. Which are the two most important cell sources contributing to cardiac development?
 9. Explain why transplantation of an avascular heart valve in a child with a congenital heart valve disorder is far from ideal?
 10. What is the difference between vasculogenesis and angiogenesis?
 11. Which process drives the specification of blood vessels in arteries and veins?
 12. Which cell types are responsible for stabilization of the vessel walls in arteries and veins and precapillaries?
 13. Schwann cells are important for survival of peripheral nerves and can be divided in two types of cells. Which types?
 14. The survival of Schwann cells critically depends on signaling molecules derived from the associated neuron. What is the name of this signaling molecule?
 15. Keratinocytes and dermal fibroblasts are derived from two distinct germ layers. Which are these germ layers?
 16. During skin development in vitro, the role of the dermal fibroblasts can be replaced by a growth factor. Which growth factor?
 17. Which three cellular processes drive embryonic skin development?
 18. Two distinct processes drive bone formation in the embryo. Name these two processes and indicate which bones are formed through either of these processes?
 19. Osteoblasts and chondrocytes at one hand and osteoclasts at the other hand are derived from two distinct cell lineages. Which are these two cell lineages?
 20. Provide two examples highlighting the critical role of environmental factors during organ formation in the embryo.

- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:
1. Describe shortly how the sequence of events that lead to the formation of the three germ layers during gastrulation can be mimicked in vitro starting from iPS cells.
 2. Epithelial to Mesenchyme Transition (EMT) is a frequent recurring process during organ formation and during tumor metastasis. Describe shortly the sequence of events that result in an EMT and provide an example of an EMT during embryogenesis.
 3. Describe the sequence of geometrical changes that lead to the formation of a functional heart.
 4. Argue why tissue engineering strategies relying on a modular approach are likely more successful than a top-down approach in which one attempts to engineer a whole organ in one step.
 5. Argue why knowledge of organ formation during embryogenesis can help in optimizing tissue engineering strategies.
 6. Describe shortly the sequential steps in bone formation by endochondral ossification.
 7. Argue why the healing of critical bone defects that do not heal spontaneously by using a tissue engineering strategy that relies on endochondral ossification is likely more successful than attempts of healing these bone defects using the intramembranous ossification pathway.
 8. Argue why it is not possible to differentiate an iPS cell directly into a cardiomyocyte.
 9. Describe the interactions between Schwann cells and peripheral nerves and vice versa.
 10. What would be your strategy to derive a differentiation protocol for the generation of kidney epithelial cells starting from an established iPS cell line?

Challenge-based learning

Engineering the next generation of functional kidney organoids

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Kidney organoids can be used as kidney replacement therapy in patients with kidney failure. Even though kidney organoids derived from iPSCs can self-organize into structures that resemble fetal kidney, several limitations still exist, namely the highly variable differentiation of iPSCs into kidney cell types, the lack of functional vascularization within the organoid, the failed formation of collecting ducts connected to nephrons, and a functional ureter. Therefore, there is a need to reduce the demand of organ donation or the use of dialysis in patients with kidney failure via organoid technology. The long-term vision is to develop a

kidney organoid that can perform diverse kidney functions including filtration of blood, secretion of endocrine and immunologic factors, reabsorption of water and electrolytes, and metabolism of minerals and nutrients.

Motivation and stakeholders

Chronic kidney disease affects about 13% of the global population. Due to the lack of regenerative capacity in the adult kidney, chronic kidney disease can progress to kidney failure that, in its late stages, can be treated only with dialysis or kidney transplantation. Dialysis is very costly and far from optimal because the patient is constantly bound to a machine. Additionally, there is shortage of kidneys for donation. As a result, millions of deaths occur every year because of kidney failure. Solutions to mitigate this problem should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as clinicians involved in precision medicine, patients, and biotech

Challenge-based learning—continued

companies and research laboratories involved in organoid manufacturing.

Problem definition

The human kidney involves an intricate relationship of 26 different cell types to form the functional organ. Current strategies in creating kidney organoids involve the induction of iPSCs on natural or synthetic matrices with a very imprecise control over cells' differentiation. This imprecise control results in high variability in iPSC's phenotype and kidney organoid architecture and, therefore, it will impact greatly on organoid's function. There is a need to improve one of the following three aspects of kidney organoid engineering, namely: (a) the induction of iPSCs to form metanephric mesenchyme, (b) the induction of nephron progenitor cells, and/or (c) the genome editing of iPSCs for developing kidney organoids for precision medicine.

Challenge

To propose a new engineering approach to generate a functional kidney organoid taking in consideration—one or a combination of—the needs mentioned above.

Learning framework

Reading the Embryogenesis chapter and relevant literature will help you to understand the following:

1. The role of the kidney in human physiology. List various functions performed by the kidney and its importance in maintaining homeostasis.
2. The embryologic developmental steps to generate an adult kidney. Include the cell types that make up this organ and how do they cooperate to make a functional kidney.

3. Outline the signaling pathways and associated factors responsible for kidney development and maturation. Indicate what key factors play the most important role(s).
4. Highlight the kidney functions that cannot yet be integrated in kidney organoids using iPSCs.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the functional aspect you want to improve in this challenge:

5. The definition of an organoid and how it can be engineered/manufactured. List and explain the key components of a kidney organoid.
6. Define bottleneck(s) in developing a kidney organoid and what technical/engineering/biological limitations prevent researchers to create a kidney-like replica.
7. Highlight the state of the art in engineering kidney organoids.
8. Define and understand the key approaches taken by scientists to engineer functional tissues.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

3.10 Glossary

Angioblast is a type of endothelial precursor cell derived from mesoderm.

Angiogenesis is the physiological process through which new blood vessels form from preexisting vessels.

Anterior is the Latin placeholder for a body part that lies in front of another, similar body part.

Apical is an anatomical term of location denoting the area of an epithelial or endothelial cell that faces the blood or intestinal fluid.

Articular chondrocytes are specialized cells of articular cartilage that are in charge of the development, maintenance, and repair of its extracellular matrix.

Autocrine is a phenomenon where cells produce a substance that has an effect on the same cell secreting said substance.

Basement membrane is a special type of extracellular matrix that lines the basal side of epithelial and endothelial tissues. The basal side opposes the apical side of these cells.

Basolateral is an anatomical term of location referring to an item situated below and toward the side.

Body plan is a set of morphological features common to many members of a phylum of animals.

Canaliculi are microscopic canals between the lacunae of ossified bone

Cell homing is a phenomenon where cells migrate toward target tissue sites prior to their proliferation and expansion.

Chondrocyte is a metabolically active cell found in the cartilage that synthesizes and degrades a large volume of extracellular matrix components.

Developmental engineering is a tissue engineering process design strategy in which lessons from developmental biology are used to develop rules for in vitro tissue development.

Dorsal is an anatomical term referring to the back or upper side of an organism. The dorsal side lies opposite to the ventral side.

Ectoderm is the outermost of the three primary germ layers in early embryonic development. It originates from the outer layer of germ cells and is the precursor of the skin and neural system and associated organs like the eye and the neural crest.

Embryogenesis is a complex and sequential series of cell division and growth events leading to the development of an embryo.

Endochondral ossification is one of the two essential processes by which bone tissue is created, in this case by ossification of a chondral precursor tissue.

Endoderm is the innermost of the three primary germ layers in early embryonic development and is the precursor of organs such as the gastro-intestinal tract.

Extracellular matrix is a three-dimensional network consisting of extracellular macromolecules, such as collagen, enzyme, and glycoproteins that provide structural and biochemical support to surrounding cells.

Gastrulation is a phase early in embryonic development where a single-layered hollow sphere of cells called blastula is reorganized into a multilayered structure through cell migration.

Gestation(al) is the time between conception and birth.

Intramembranous ossification is the direct conversion of mesenchymal tissue into bone.

Mesoderm is the middle of the three primary germ layers in early embryonic development. Precursor of muscle and connective tissue, cartilage, bone, notochord, blood, bone marrow, lymphoid tissue, and the epithelia (surface, or lining, tissues) of blood vessels, lymphatic vessels, body cavities, kidneys, ureters, gonads (sex organs), genital ducts, adrenal cortex.

Modularity of the tissue architecture is a phenomenon where several units of tissue intermediates are combined to form a larger tissue.

Morphogens are signaling molecules that act directly on cells to produce specific cellular responses depending on their local concentration.

Mural cells are vascular smooth muscle cells that provide support to blood vessels.

Neural crest cells originate between the neural plate and nonneural ectoderm during embryonic development.

Nucleus pulposa is a soft and gelatinous inner core of the vertebral disc that moves within the disk with changes in posture.

Organogenesis is the process by which the embryonic cells of the three germ layers develop into organs.

Osteoblast is a specialized mesenchymal cell that synthesizes bone matrix and coordinates the mineralization of the skeleton.

Osteoclast is a giant cell containing between 10 and 20 nuclei, involved in remodeling bone matrix.

Paracrine is a type of cellular communication in which a cell produces a signal to induce changes in nearby cells, altering their behavior. Factors are secreted into the immediate extracellular environment.

Pluripotent refers to the property of cells that make them capable of giving rise to cells of the ectoderm, mesoderm, and endoderm lineages.

Primary ossification center is the first area during endochondral ossification to start forming bone tissue.

Remak fibers are a class of nerve fibers that carry sensory information and are unmyelinated.

Secondary ossification center is the area of the ossification center that appears after the primary ossification center.

Systemic factors are substances that affect the whole body and not specific tissues/organs.

Trophic (factors) are substances that allow a cell to grow.

Vasculogenesis is the process of blood vessel formation in the embryo, occurring by de novo production of endothelial cells.

Vernix caseosa is a white, creamy, naturally occurring biofilm covering the skin of the fetus during the last trimester of pregnancy.

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Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

Cellular signaling

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4.1 Learning objectives

After reading this chapter you will be able to:

- Describe the paradigm of cell signaling, which incorporates signal initiation, transduction, and gene activation.
- Appreciate the complexity of signaling and give examples of deviations from the paradigm.
- Describe how G-protein–coupled receptors and tyrosine kinase receptors transmit signals.
- Know the major steps of TGF- β , Wnt, Rho kinase, NF κ B, and vitamin D signaling.
- Understand how the tissue environment sets the context for signal transduction.
- Be aware of how cell signaling can be modulated to improve outcomes for tissue engineering.

Does matrix produce a specific diffusible chemical agent that induces the cells of the host to differentiate into osteoblasts? The answer is no. The system is more complex than a simple chemical stimulus and direct cell response ...

Urist MR. Bone: formation by autoinduction. *Science*. 1965;150:893–899

The idea that such complexity can arise not only out of such simplicity, but probably absolutely out of nothing, is the most fabulous, extraordinary idea.

D. Adams.

4.2 Paradigm of cellular signaling

Tissue engineering aims to recapitulate dynamic biological processes such as development and regeneration. For these processes to occur successfully, cells must receive the right combinations of signals in the right concentrations at the right time. Tissue engineers should take comfort in the fact that all cells are conferred with the core machinery (e.g., their DNA and the molecules for transcription and translation) needed to behave the way we want them to behave. The challenge is to modulate the molecular mechanisms, referred to as **cellular signaling**, by which cells respond to their environments and communicate with each other. By controlling cellular signaling, we can influence cell behavior, which is ultimately the goal of tissue engineering.

In the first section of this chapter, some general principles of cellular signaling will be outlined and further illustrated using specific examples from major classes of signaling pathways. Examples will be given of tissue engineering strategies that target these pathways in order to evoke specific responses from cells. The reader is encouraged to refer to the later chapters of this book for cell signaling knowledge relevant to specific tissues.

It is important to note that due to the sheer number of molecules involved in signaling and the complexity of their interactions, there will be many exceptions to the general rules outlined here. But there are basic paradigms that can be understood. For example, much of cellular signaling is initiated by the generation of a **ligand**, which is secreted by a sending cell or bound within the extracellular matrix, to bring about a change in the physiology of a responding cell (Fig. 4.1A). The repertoire of proteins expressed by the responding cell (which are determined by the state and the environment of that cell) determines whether and how it responds to a certain signal. This typically involves the binding of the ligand to a receptor to initiate a cascade of biochemical changes that result in immediate changes to proteins and ends in the transcription of genes. Possible responses include altered survival, proliferation, differentiation, migration, cytokine production, metabolism, cytoskeletal rearrangement, and extracellular matrix production—all of which are important for tissue engineers.

A fundamental lesson to take from this chapter is that cellular signaling is astonishingly complex. For instance, since the gene and protein expression profile are different for every cell type, different cells will respond differently to the same signals. Examples abound, just one of which is the cellular response to the glucocorticoid hormones, which trigger cell death in lymphocytes, but stimulate osteogenic differentiation in mesenchymal stem cells. Furthermore, cells respond differently to ligands at different concentrations and different rates of change. They also respond differently depending on their environment and their individual history. To add to this complexity, cells in the body are not exposed to a single signal at a single concentration, but to a cocktail of hormones, cytokines, and growth factors, which can integrate their signals in unexpected ways. And finally, not all signaling begins with a ligand–receptor interaction. Of particular relevance to tissue engineering, cell signaling can also be initiated

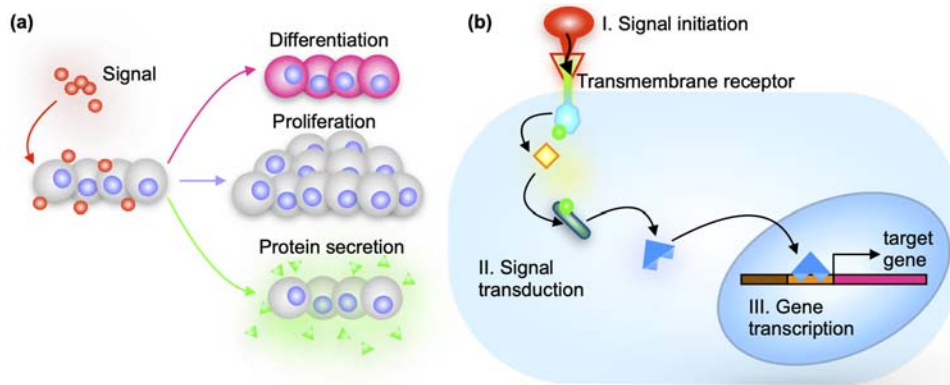


FIGURE 4.1

(a) Cell signaling leads to changes in the responding cell. (b) Although many variations on this theme exist, signaling events often adhere to this paradigm.

by physical cues, such as the elasticity of the substrate upon which a cell resides (see [Chapter 5 Extracellular matrix as a bioscaffold for tissue engineering](#) and [Chapter 6 Synthetic Biomaterials](#)). Nonetheless, our present understanding is detailed enough to be applicable by tissue engineers aiming to direct cell behavior, and many studies have shown that mimicking the ligand–receptor interactions that exist in native tissue can improve the behavior of cells in a tissue-engineered construct.

A central paradigm can be recognized in most events of cellular signaling, consisting of three distinct steps ([Fig. 4.1B](#)):

- I. Signal initiation: An extracellular ligand binds to a receptor on the surface of the cell. Ligand binding changes the activity or conformation of the receptor, thus transducing the signal to the inside of the cell.
- II. Signal transduction: The activated receptor triggers a signal transduction cascade in which intracellular proteins are activated, ultimately leading to activation of a transcription factor in the nucleus.
- III. Gene activation: The transcription factor binds to regulatory sequences in target genes, resulting in gene activation, protein synthesis, and changed cellular physiology.

In the following section, some main types of signals, receptors, and the machinery for gene activation are described. Examples from specific tissues and tissue engineering are given, but it is important to note that many of these pathways are involved in multiple cell types.

4.3 Signal initiation

The first step in this paradigm of cellular signaling is the interaction of a ligand with its receptor, but even before that happens, a context for the signaling event has been established in the receiving cells through the genes and proteins they express. This is one way by which the microenvironments designed by tissue engineers can have such a profound effect on cell behavior. For example, whether cells are cultured as monolayers or aggregates, the extracellular matrix components they may be grown on, and the media used for culture can all prime the cell to receive and integrate an incoming signal differently.

A wide variety of molecules in the body serve as ligands and these can be incorporated in tissue engineering strategies to influence cell behavior. Most ligands share the characteristic that they physically interact with their receptor, which is typically a transmembrane protein that binds with the signal molecule in the extracellular space and transduces the signal into the cell. This is certainly the case for hydrophilic ligands or molecules that cannot pass through the plasma membrane, but some small or hydrophobic molecules can diffuse through the membrane and bind receptors or targets in the cytosol or nucleus.

Generally, ligands can be categorized into the following:

- Extracellular matrix proteins
- Membrane-bound proteins
- Diffusible molecules

Extracellular matrix proteins act as a ligand to enable cells to sense and respond to the composition of their environment, an event that primarily occurs via a class of receptors known as the integrins (see [Chapter 8 Cell–material interactions](#)). Many extracellular matrix proteins are ligands for specific receptors. For example, cells can detect whether they are surrounded by collagen and produce the correct enzymes to remodel their surroundings (see [Chapter 5 Extracellular matrix as a bioscaffold for tissue engineering](#)).

Membrane-bound proteins act as ligands to enable cells to determine whether they are in contact with each other, and to gain information about a neighboring cell. An impressive example of the power of membrane-bound proteins to influence cell behavior is the way by which cadherins, a diverse family of transmembrane proteins, enable similar cells to stick together and segregate themselves from other cell types. With cadherins, cells can organize based on both a quantitative (i.e., the amount of a cadherin) and qualitative (i.e., the member of the cadherin family) basis. For example, if cells from two tissues such as the heart (expressing N-cadherin) and the skin epithelium (expressing E-cadherin) are dissociated and mixed together, cadherins can guide them to form two distinct populations. Beyond tissue organization, cell–cell contact can inform diverse changes, such as telling cells within a tissue when to stop growing (through the Hippo pathway) or trigger an immune response.

Diffusible molecules are produced when a sending cell produces a protein (e.g., a growth factor) or organic substance (e.g., a steroid hormone) and secretes it into its environment where a responding cell (which can be the same cell that produced the ligand) presenting a receptor binds it. While tissue engineers are mainly focused on growth factors, other diffusible molecules include amino acids, nucleotides, and dissolved gases, some of which can enter the cell and bind to an intracellular receptor. Hydrophobic compounds, such as steroid hormones, can also pass through the membrane and initiate signaling from inside the cell.

It is important for tissue engineers to consider the **length scale** by which they wish to influence cell signaling. For example, while extracellular matrix and membrane-bound proteins act on cells in physical proximity to the ligand, the action range of diffusible molecules varies. In the body, they can be short range, such as the interleukins (ILs) produced in the dermis to control epidermal signaling or long range (endocrine), such as the production of the parathyroid hormone in the parathyroid glands that affects bone remodeling. In some cases, a cell will produce and retain a signaling ligand intracellularly or at its own surface membrane, where it activates signaling within the producing cell, a phenomenon known as autocrine stimulation. Tissue engineers can modulate these ranges by, for example, drug delivery strategies (see [Chapter 12 Controlled release strategies in tissue engineering](#)) and coupling of molecules to biomaterials.

In addition to developing strategies to provide ligands to cell receptors, tissue engineers can also improve upon the activity of a ligand. For example, insulin-like growth factor-1 was fused with a protein fragment that improved its incorporation into a fibrin matrix. When implanted in a bladder lesion in rats, the engineered growth factor acted to increase the proliferation of the smooth muscle cells to improve regeneration [1].

4.4 Signal transduction

Cell signaling leads to biochemical changes to proteins (Fig. 4.2). When these changes occur to proteins that are already present in the cell, they occur quickly. However, signals that require changes in gene expression and translation of new proteins occur more slowly and often involve a more complex series of events. The complexity and specificity of signaling pathways are both largely due to the cascades they initiate. These cascades allow different ligand–receptor interactions to converge on the same pathway, or for the same ligand–receptor interaction to initiate different pathways depending on factors like the cell type or the extracellular environment.

When the endpoint of signaling is transcription, the signal initiated by the ligand–receptor interaction has to be transduced from the membrane to the nucleus. Different ligand–receptor combinations can trigger different cascades of molecular interactions in the cytoplasm, resulting in the relay of the signal. In many cases, the signal is conveyed by direct protein–protein interactions and subsequent modification of target proteins, leading to their activation. For example, binding of bone morphogenetic protein 2 to its receptor leads to activation of the receptor’s serine/threonine **kinase** activity and phosphorylation of target proteins. Phosphorylation is a common biochemical event that acts as a cellular signal. Some of the most prevalent protein modifications are summarized in Table 4.1. Alternatively, activated proteins drive the production or relocalization of the so-called **second messengers**, which are small molecules such as cyclic AMP, phospho-inositol-2-phosphate, calcium, and nitric oxide, which act as ligands for target proteins.

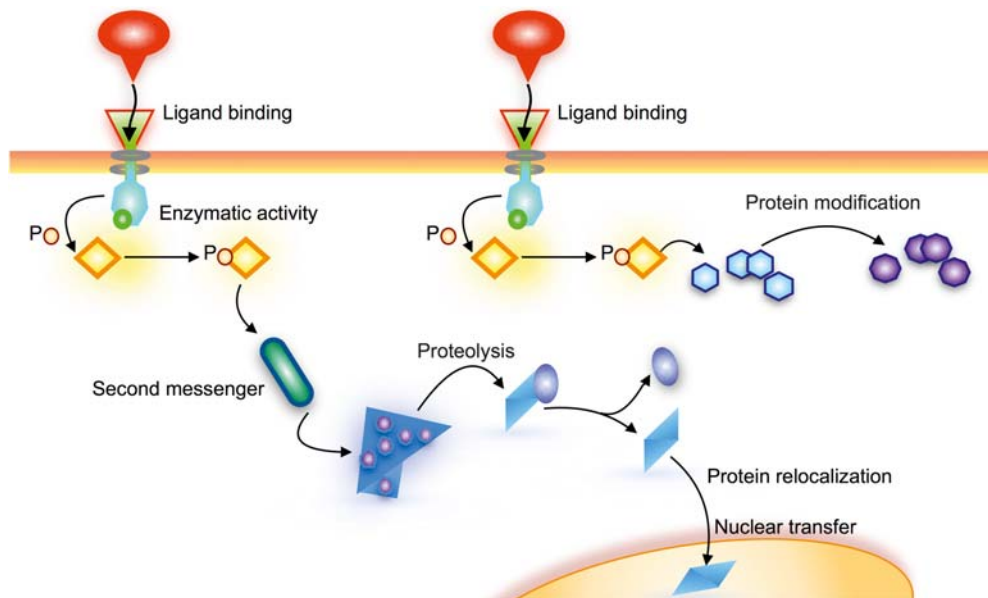


FIGURE 4.2

A hypothetical signaling pathway with some of the most relevant molecular mechanisms involved in signal transduction.

Table 4.1 Enzymatic activities involved in signal transduction.

Enzyme	Activity	Putative effect
Kinase	protein phosphorylation	Changed enzymatic activity or protein stability
Phosphatase	protein dephosphorylation	Changed enzymatic activity or protein stability
Ubiquitin ligase	protein ubiquitination	Protein degradation
Proteases	protein site-specific cleavage	Degradation, activation
Fatty acid esterase	palmitoyl/myristoyl addition to protein	Relocalization to the plasma Membrane
SUMO ligase	protein SUMO modification	Protein degradation
Lipase	Cleavage of phospholipids	Generation of second messengers, such as PIP2 and IP3
Cyclase	Forms a cyclic compound	Generation of second messenger cAMP and cGMP

A single **signaling cascade** will typically contain many types of proteins. It might begin with a ligand–receptor interaction that changes the conformation of the receptor and causes it to recruit enzymes. The next steps may involve posttranslational modifications, amplification steps, the convergence or divergence of the cascade, and ultimately result in a transcription factor entering the nucleus and binding DNA in consort with cofactors or other transcriptional modulators.

Generally, protein activity can be controlled in several ways. The enzymatic activity of the protein can be switched on, as with BMP receptor activation. This is one mechanism of signal amplification, as a single activated protein can catalyze the modification of many others. Furthermore, proteins can be sequestered away from their site of action as seen, for instance, with the transcription factor NF- κ B, which is held in the cytoplasm by the inhibitory protein I κ B until upstream signaling events allow its translocation to the nucleus. Another way to control protein activity is its abundance. Cell signaling can, in some cases, inhibit the constitutive proteolytic degradation of a certain protein, which then accumulates and exerts its function.

Importantly, signaling cascades have feedback loops, which can attenuate or amplify the signal. In a pathway with positive feedback, the outcome of one step increases its own production, thereby increasing the response to the signal. Alternatively, in a pathway with negative feedback, the output inhibits its own production, thereby preventing a response to a ligand. In reality, these feedback loops are more complicated, and are responsible for phenomena such as the oscillation of an enzyme in response to sudden changes in the concentration of a ligand.

Likewise, signaling cascades can have highly nuanced behavior during transduction. For instance, the opposing activities of kinase and **phosphatase** enzymes can act on the same protein, where the final phosphorylation state is often the result of these opposing activities. This balance occurs for most enzyme-mediated cascades in cellular signaling. There is also nonlinear behavior in signaling pathways, where the generation of a product through an enzymatic reaction can reduce or increase further product formation. This type of nonlinear signaling is often on account of enzyme adaptor domains that can direct localization in the cell. For instance, a phosphorylated residue of a protein can serve as a binding

site for a kinase enzyme, thus bringing additional kinase enzymes into close proximity for subsequent phosphorylation events in neighboring proteins. This type of autocatalytic cascade has been observed in receptor tyrosine kinase (RTK) signaling.

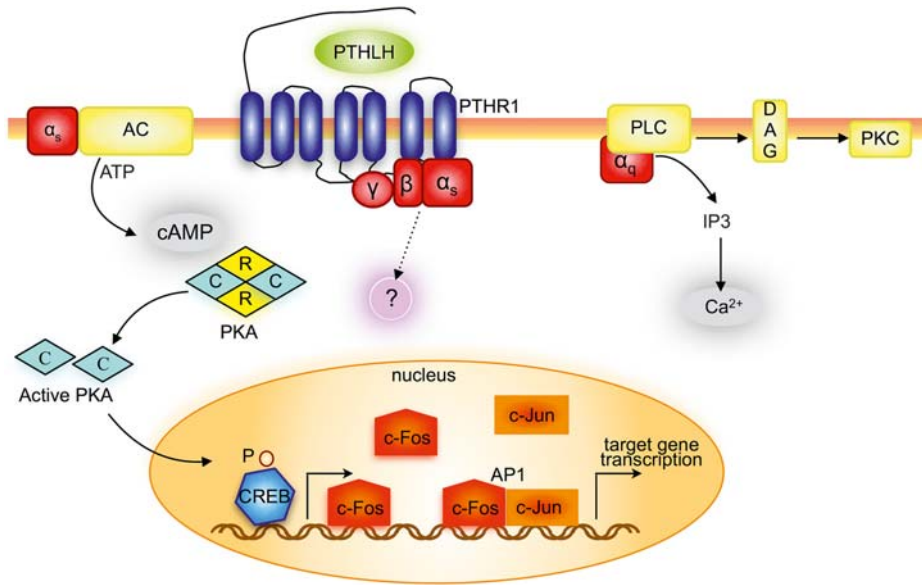
This is the overall paradigm of how cell signaling can work. In the rest of this section, a succinct overview of some specific cellular signaling pathways will be given.

4.4.1 G-protein—coupled receptor—mediated signaling

G-protein—coupled receptors (GPCRs) are the largest family of cell surface receptors. This family contains many different receptors that play an important role in physiology, including the adrenergic receptor, light and odor receptors, and receptors for small peptide hormones, such as vasopressin and glucagon, and neuropeptides such as serotonin. All GPCRs have a common structure: they consist of a single amino acid chain containing an N-terminal extracellular domain, a transmembrane domain in which the amino acid chain traverses seven times through the cell membrane, and a cytoplasmic C-terminus. The N-terminus and the extracellular amino acids of the transmembrane domain are involved in binding of the ligand, while the cytoplasmic amino acids of the transmembrane domain and the C-terminus are involved in signal transduction [2].

All G-protein—coupled receptors transduce their signal via heterotrimeric G-proteins, consisting of an α -, β -, and γ -subunit. In the absence of a ligand, the α -subunit, a GTPase, binds guanosine diphosphate (GDP) and the G-protein is inactive. Binding of a ligand induces a conformational change in the receptor, resulting in the release of GDP from the α -subunit and the binding of guanosine triphosphate (GTP) in its place. Next, the activation causes a dissociation of the α -subunit from the β - and γ -subunits. Once dissociated, the α -subunit and the $\beta\gamma$ complex are active and available for signaling. A second mechanism of action is thought to be that the **conformational change** results in the exposure of previously hidden binding sites residing between the α -subunit and the $\beta\gamma$ complex, which are targets for enzymes or ion channels.

The parathyroid hormone—like hormone is a small peptide hormone that binds with high affinity to the type 1 parathyroid hormone receptor (PTH1R; Fig. 4.3), a member of the GPCR superfamily. This hormone is important for bone and cartilage development and has thus been a target for tissue engineers. GPCRs generally transduce their signal through one class of α -subunits: $G_{\alpha s}$, $G_{\alpha q}$, $G_{\alpha i}$, or $G_{\alpha 12/13}$, but some receptors, like PTH1R, can activate two classes of α -subunits ($G_{\alpha s}$ and $G_{\alpha q}$). $G_{\alpha s}$ subunits are involved in the activation of **cyclases**, which are anchored in the cell membrane. Cyclases convert nucleotide triphosphates into cyclic nucleoside monophosphates, which act as second messengers (intracellular signaling molecules). Activation of the PTH1R results in the release of the $G_{\alpha s}$ subunit and the activation of adenylate cyclase, which converts ATP into cAMP. Subsequently, cAMP binds to the regulatory subunit of cAMP-dependent protein kinase A (PKA), thereby removing its inhibitory actions on the catalytic subunit of PKA. The newly available catalytic subunit of PKA then phosphorylates serine residues on proteins. One of the main nuclear targets of PKA is the transcription factor cAMP response element binding (CREB) protein. This transcription factor is bound in an inactive form to specific DNA sequences present in promoters of a wide variety of genes until PKA activates it by phosphorylation of serine-133, resulting in the start of gene transcription. Of interest for

**FIGURE 4.3**

G-protein—coupled receptor signaling.

tissue engineers, the cAMP/PKA pathway can be activated by various chemical substances such as forskolin, which is an activator of adenylate cyclase, and isobutyl methyl xanthine, which is an inhibitor of cAMP degradation. These molecules make it possible to modulate cell signaling without using protein ligands.

4.4.2 Receptor tyrosine kinase signaling

RTKs are a major class of **transmembrane receptors** that are responsible for binding many extracellular and cell surface proteins and initiating the subsequent signaling pathways. The extracellular ligands include many relevant proteins in morphogenesis and regeneration, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGFs), platelet-derived growth factor (PDGF), nerve growth factor, and insulin. RTKs generally work by **autophosphorylation** upon ligand binding, which causes dimerization of the receptor such that the kinase domain on one receptor can phosphorylate its neighbor. The subsequent signaling steps can lead to diverse changes in cell behaviors relevant to tissue engineering, such as morphogenesis, survival, proliferation, and migration, among others.

One highly relevant example for tissue engineering are the receptors that act during vascular morphogenesis. These include the VEGF receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), PDGF receptor- β (PDGFR β), and the angiopoietin receptor, Tie-2. The VEGF receptors are primarily involved in the early stages of blood vessel assembly and providing soluble VEGF is a widely used strategy for improving vascularization in tissue engineering. Their activation upon ligand binding leads to the migration, proliferation, and eventually the differentiation of endothelial cells to form a new, lumen-containing vessel.

The binding of VEGF to VEGFR-2 can lead to the activation of several signaling pathways involved in angiogenesis, one of which is the mitogen-activated protein kinase (MAPK) pathway (Fig. 4.4). The binding activates the tyrosine kinase activity of the cytoplasmic domain of the receptor, providing a binding site for the adapter protein Grb2 that in turn localizes Sos to the plasma membrane. Sos activates Ras by replacing GDP with GTP. Ras-GTP binds directly to a serine-threonine kinase, Raf, forming a transient membrane-anchoring signal. Active Raf kinase phosphorylates a dual specificity kinase, mitogen-activated kinase (MEK), and activates it. The activated MEK phosphorylates ERK1/ERK2, which subsequently translocates to the nucleus, where it can phosphorylate the transcription factor Ets-1. Ets-1 then regulates the expression of multiple angiogenic genes like MMP-1, MMP-9, integrin β 3, and VE-cadherin [3].

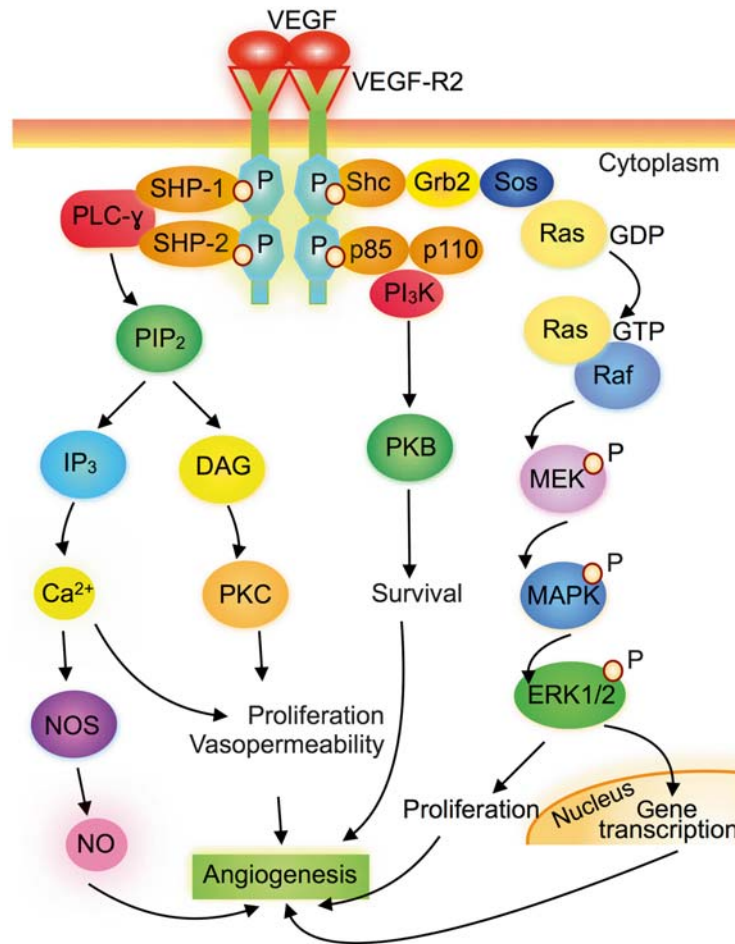


FIGURE 4.4

Binding of VEGF to its receptor leads to the activation of several signaling pathways that are involved in angiogenesis.

The addition of growth factors like VEGF to engineered tissues could initiate faster vessel formation after implantation. However, in order to get stable, functional vascular structures, other factors are necessary as well. For example, a sequential release approach was taken to supply two growth factors to improve vascularization. PDGF-BB, which signals smooth muscle cells to stabilize vessel walls, was combined with VEGF-A inside a hydrogel. The two growth factors had two different release profiles, a strategy that improved cardiac function after a myocardial infarction in rats [4,5]. Therefore, a thorough understanding of RTK signaling pathways involved both in initial vessel formation and vessel maturation is important to be able to enhance the vascularization of engineered tissues after implantation.

4.4.3 TGF- β superfamily signaling

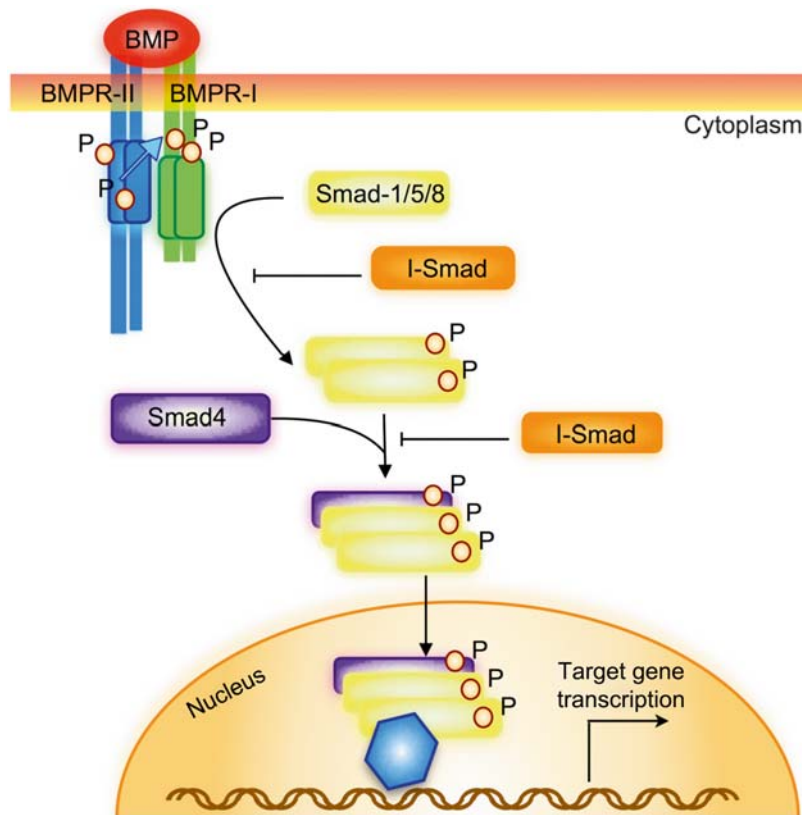
TGF- β signaling is a central pathway in both tissue development and homeostasis. The TGF- β superfamily consists of various subfamilies, including TGF- β s, BMPs, activins, inhibins, and the anti-Müllerian hormone. TGF- β signaling displays some remarkably complex regulation. For example, when a TGF- β receptor is bound to its ligand, it is **endocytosed** and either further activated or inactivated depending on the means of endocytosis. Activation depends on clathrin-coated vesicles and the formation of endosomes, inside of which the Smad-dependent pathway continues. Inactivation depends on caveolae and is followed by ubiquitination of the receptor leading to its degradation [6].

Here we focus on the major group of the TGF- β superfamily, the BMPs, which are secreted growth factors that were originally identified by their ability to induce ectopic bone and cartilage formation [7] making them highly relevant to tissue engineers. The BMP subfamily consists of more than 20 members. BMPs exert their effects through distinct combinations of two different types of serine/threonine kinase receptors: type I receptors and type II receptors. Receptor activation involves ligand-induced hetero-oligomerization of two sequentially acting kinases, with the type I receptor acting as a substrate for the type II receptor kinase (Fig. 4.5). The activated, phosphorylated type I receptor then propagates the signal through phosphorylation of receptor Smads (R-Smads-1, -5, and -8 for BMP and -2 and -3 for TGF) that assemble into heteromeric complexes with Co-Smad4 and translocate to the nucleus, where they regulate transcription of target genes. In addition, BMPs stimulate other pathways that are distinct from the Smad pathway, such as activation of Jnk and p38 MAP kinase pathways [8].

Negative regulation of BMP activity occurs at nearly every step in the BMP/Smad pathway. Intracellularly, inhibitory Smads (I-Smad6 and 7) antagonize R-Smads by competing with them for interaction with activated receptors or **heteromeric complex formation** with Co-Smad4 (Fig. 4.5). I-Smads also prevent activation of R-Smads by recruitment of **ubiquitin** ligases, like Smad ubiquitination regulatory factor 1 (Smurf-1) and Smurf-2, which add a ubiquitin moiety to Smad proteins, earmarking them for destruction by the proteasome.

Extracellularly, soluble pseudoreceptors and antagonists inhibit BMP activity. Extracellular antagonists include Noggin, Chordin, DAN, Cerberus, and Gremlin, which are thought to operate by blocking binding of BMPs to their receptor [9].

In tissue engineering, strategies can be designed to control BMP signaling activity at each of the regulatory steps, both outside and inside the cell. Human embryonic stem cells could be differentiated

**FIGURE 4.5**

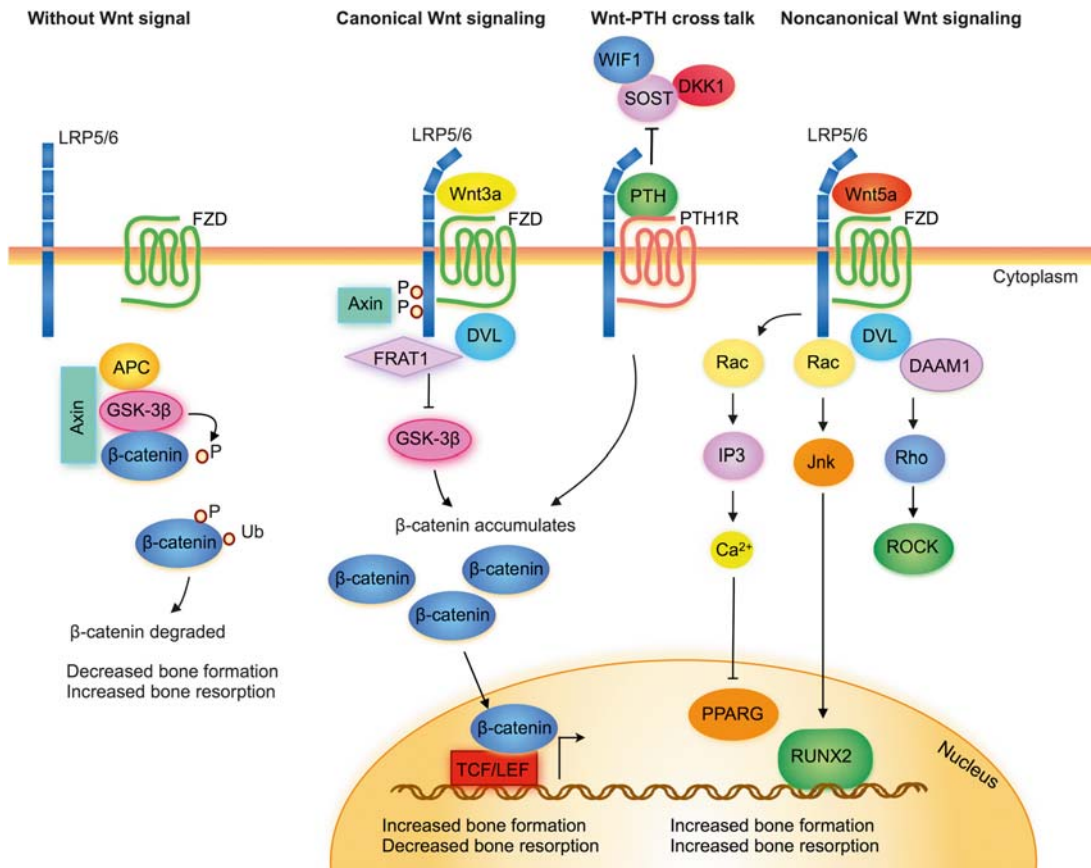
A model for activation of bone morphogenetic protein (BMP) receptors.

into chondrocytes by induction with BMP-7 and TGF- β 1 [10], and the controlled release of TGF- β 1 from a hydrogel scaffold activated Smad and ERK1/2 pathways in mesenchymal stem cells and improved their chondrogenic differentiation both in vitro and after implantation [11].

4.4.4 Wnt signaling

Like BMPs, Wnts (pronounced as “wints”) are secreted signaling molecules with pivotal roles in a variety of cellular activities important for tissue engineers, including cell fate determination, proliferation, differentiation, migration, and polarity. Wnt signaling is an example of a pathway that depends on **regulated proteolysis**, a mechanism that enables cells to respond rapidly to an extracellular signal.

There are 19 Wnts in humans that all bind to a receptor of the Frizzled family, sometimes in conjunction with other membrane-associated proteins, such as low-density lipoprotein receptor–related proteins (LRPs). Frizzled then recruits Dishevelled, a scaffold protein that initiates the signaling cascade. There are three major signaling pathways that Wnts can activate: (1) β -catenin (also known as canonical Wnt signaling), (2) planar cell polarity pathways, and (3) Ca^{2+} pathway (Fig. 4.6). In canonical Wnt

**FIGURE 4.6**

A simplified view of Wnt signaling in bone homeostasis. Adapted from Baron R, Kneissel M. *Wnt signaling in bone homeostasis and disease: from human mutations to treatments*. Nat. Med. 2013;19:179–192.

signaling, LRP5 and LRP6 act as coreceptors and upon Wnt binding, intracellular β -catenin is stabilized and thereafter enters the nucleus where it interacts with **transcription factors** in the TCF/LEF family. The key to this latent regulation mechanism is that without Wnt signaling, a group of proteins form a degradation complex that binds and phosphorylates β -catenin, marking it for ubiquitination and degradation in proteasomes. In noncanonical Wnt signaling, pathways are regulated through means outside of the β -catenin localization mechanisms and are often initiated through interactions with the extracellular matrix. For instance, in the planar cell polarity pathway, activation of Frizzled regulates the polarity of cells by affecting their cytoskeletal organization. In the Ca^{2+} pathway, activation of a heterotrimeric G-protein results in an increase in intracellular Ca^{2+} and activation of calcium/calmodulin-regulated kinase II and protein kinase C. Extracellularly, the Wnt inducible factor 1 and the secreted Frizzled-related proteins antagonize Wnt signaling by interfering with the functional interaction between Wnts and their Frizzled receptors [12]. Using another mechanism, Dickkopf proteins compete for the LRP5 and LRP6 coreceptors to prevent activation of the canonical signaling pathway.

The canonical Wnt signaling pathway is important for bone homeostasis and the pathway represents a therapeutic target for patients at risk for fractures related to aging and disease [13]. Scientists aiming to control the differentiation of stem cells also take advantage of small molecule modulators, such as CHIR99021, that inhibits glycogen synthase kinase 3, which itself is an inhibitor of the Wnt pathway. In this way, this small molecule can activate canonical Wnt signaling. On the other hand, noncanonical Wnt signaling plays roles in guiding cellular organization and patterning during development and signaling through the planar cell polarity or Ca^{2+} pathway is often initiated by extracellular signals like integrin engagement of the surrounding matrix. Thus, understanding how the physical properties of tissue guide these paracrine and autocrine signaling pathways is important for tissue engineering.

4.4.5 Rho kinase signaling

A number of ligand–receptor interactions (GPCRs, RTKs, integrins, cadherins, and others) can lead to changes in Rho kinase signaling, and it is worth a small section of its own owing to its importance in the context of tissue engineering.

Firstly, cell migration requires coordinated signaling between the protruding front of the cell and the retracting rear. It is the Rho family of GTP-binding proteins (GTPases) that play key roles in mediating cytoskeletal arrangement and migration, namely the three family members Rho, Rac, and Cdc42. Rho regulates the banding of actin filaments into stress fibers and helps in the formation of focal adhesions by clustering integrins (see [Chapter 8 Cell–material interactions](#)). Rac promotes **lamellipodia** extension through the polymerization of actin at the cell periphery. Finally, Cdc42 mediates **filopodia** formation.

Like other GTPases, the Rho family proteins are **molecular switches**, residing in an inactive state when bound to GDP and an active state when bound to GTP. Forward cell movement requires Rac activation to induce protrusions at the leading edge of the cell [14]. These lamellipodia are actin-rich adhesive structures that concentrate high-affinity integrins into focal adhesions to facilitate their binding to extracellular matrix proteins, while also actively advancing the leading edge of the cell. Rac activation is an example of a positive feedback loop, since it produces PI 3-kinase which leads to further activation of Rac. Rho affects stress fiber formation by activating formins and Rho kinases (ROCKs). ROCKs act by both inhibiting a phosphatase and by phosphorylating various downstream targets including myosin II light chain, leading to cell contraction.

For tissue engineering, the Rho-GTPase pathway has been implicated in the phenomenon known as mechanotransduction, which refers to the fact that some cell types can sense the elasticity of their substrates (see [Chapter 8 Cell–material interactions](#)). The seminal paper in this field demonstrated that mesenchymal stem cells could be induced to differentiate to a specific tissue lineage when grown on a substrate that matched the elasticity of tissue from that lineage [15]. Cells grown on a material with elasticity similar to brain, muscle, and bone osteoid differentiated into neurogenic, myogenic, and osteogenic cells, respectively. This was found to depend on nonmuscle myosin II and differentiation did not occur when blebbistatin, a nonmuscle myosin II inhibitor, or ML7, a myosin light chain kinase inhibitor, was used.

4.4.6 NF- κ B signaling

NF- κ B proteins are known as **latent regulatory proteins**, referring to the fact that they are already present in most cells, but are activated in response to stress. They are central to the inflammatory and innate immune responses, which, in the case of wound healing, are initiated by the binding of IL-1 to its receptor.

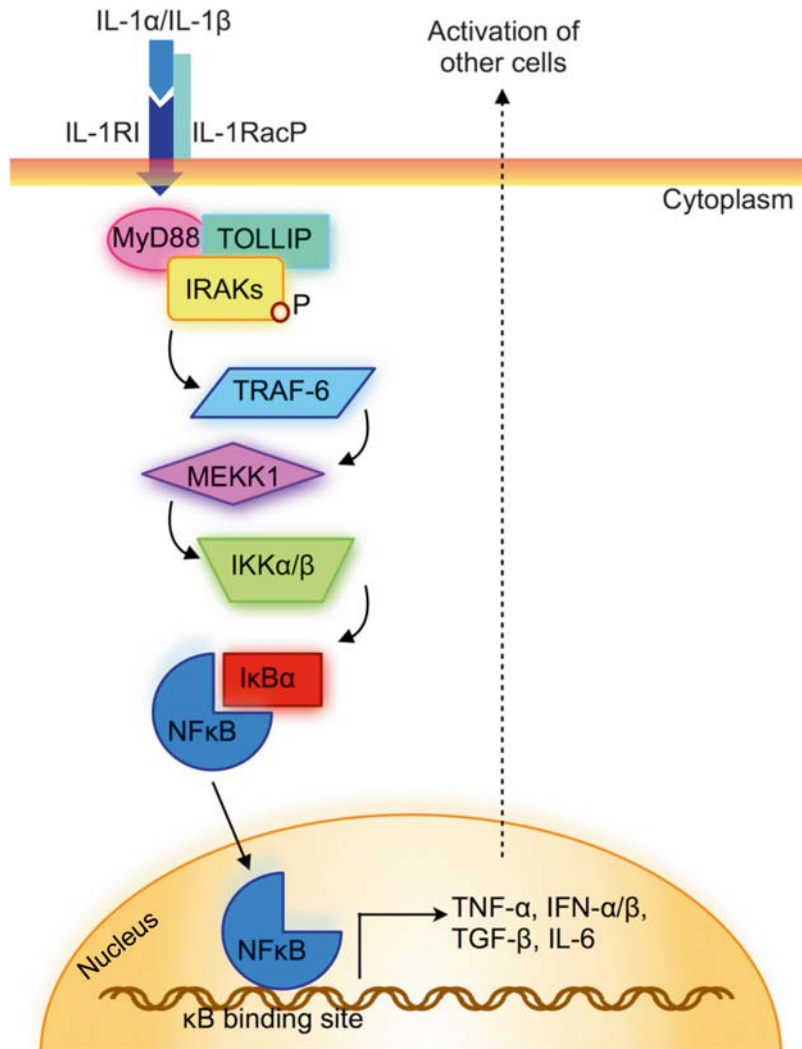
ILs are a class of proteins (**cytokines** and **lymphokines**) that are released by cells of the immune system and act as intercellular mediators in developmental regulation, tissue repair, hemopoiesis, inflammation, and specific and nonspecific immune responses. IL-1 promotes keratinocyte and fibroblast proliferation and induces the expression of intercellular adhesion molecules in endothelial cells and fibroblasts. The signal transduction events begin with the binding of IL-1 to its receptor, IL-1RI. This induces the recruitment and interaction of IL-1RacP to IL-1RI, resulting in a high affinity complex for IL-1 (Fig. 4.7). IL-1RI and IL-1RacP recruit MyD88, a member of the IL-1 receptor-associated protein kinases family, and tumor necrosis factor receptor-associated factor-6 (TRAF-6). Together, they activate the inhibitory κ B (IkK) complex, consisting of the inhibitory protein IkK and the transcription factor NF- κ B. The TRAF-6 signal phosphorylates the IkK subunit, thereby releasing the NF- κ B dimer from the complex. NF- κ B then translocates to the nucleus where it acts as a transcription factor by binding to κ B binding sites in target genes such as IL-6 and IL-8. IL-1 signaling can also lead to the activation of c-jun N-terminal kinase (Jnk) and other MAPK that result in the phosphorylation and activation of AP-1, another transcription factor that causes expression of genes related to wound healing [16].

4.4.7 Vitamin D signaling

Vitamin D signaling exemplifies a deviation from the paradigm of cell signaling because the vitamin D receptor (VDR) resides intracellularly and acts directly as a transcription factor once it is bound to vitamin D. Vitamin D levels have been implicated in many regeneration processes, one of which is its role in bone, where vitamin D is essential for the development and maintenance of a mineralized matrix and enhancement of calcium absorption. Strictly speaking, vitamin D is not a vitamin but is a hormone produced by the skin that is metabolized into more active compounds in peripheral tissues (Fig. 4.8). The compound 1,25-(OH)₂D₃, also known as calcitriol, is the active form of vitamin D with the highest binding affinity for the VDR, a member of the steroid receptor superfamily. These nuclear receptors act as ligand-activated transcription factors that interact with coregulators and the transcriptional preinitiation complex to regulate gene transcription. Binding of 1,25-(OH)₂D₃ induces heterodimerization of the VDR with the retinoid X receptor. This heterodimer binds with high affinity to vitamin D response elements in the promoters of target genes such as osteocalcin, one of the most abundant proteins in bone.

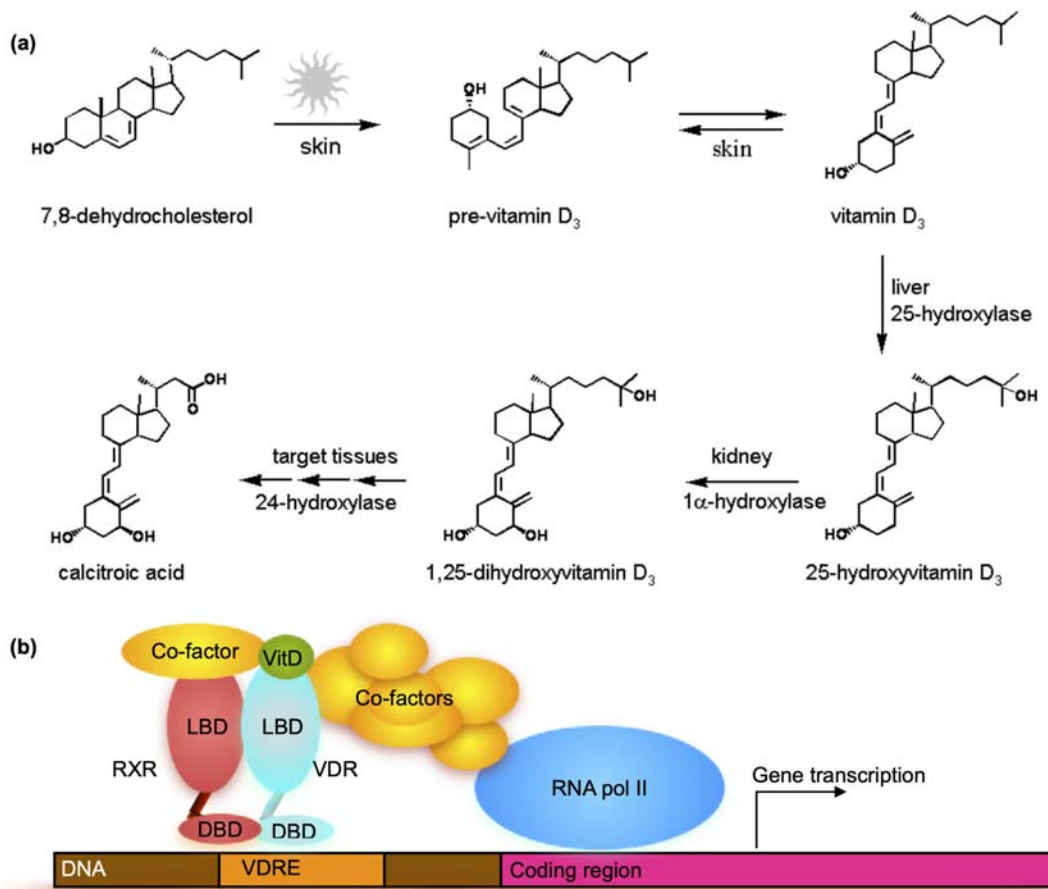
4.5 Gene activation

The last step in the signal transduction cascade is gene activation (Fig. 4.9A). Gene activity is mainly controlled by the rate of transcription of the gene, the process in which a messenger RNA is produced by RNA polymerase. Signal transduction initiates the transcription process by activating the so-called transcription factors, which are sequence-specific DNA-binding proteins. The transcription factor is either retained in the cytoplasm and is translocated upon activation or the transcription factor is already

**FIGURE 4.7**

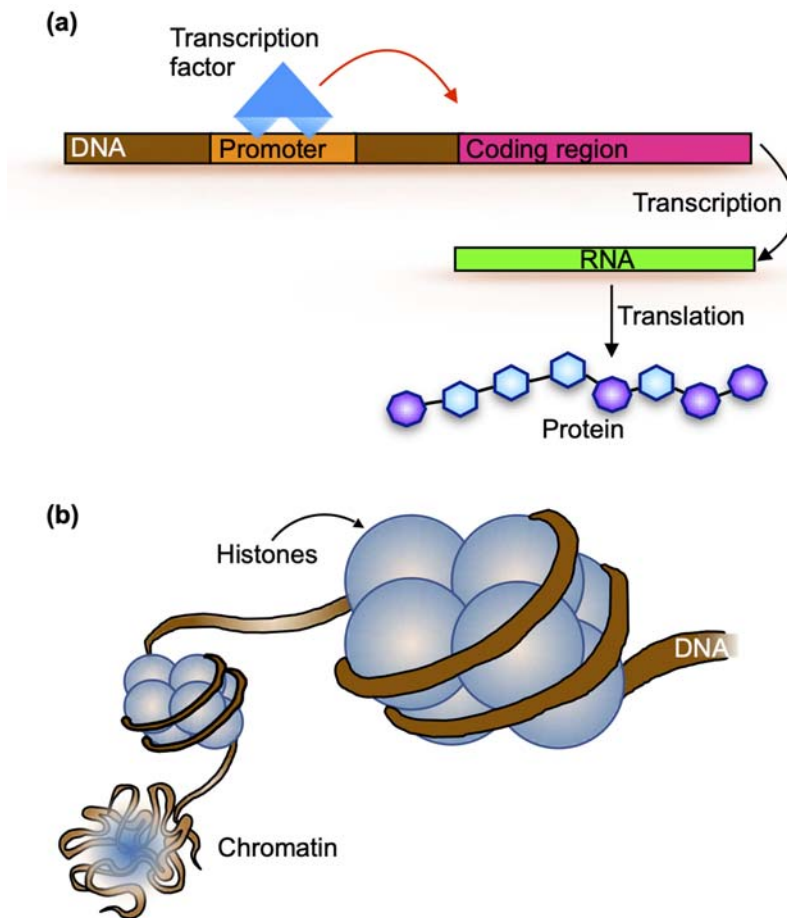
The interleukin (IL-1) signal transduction pathway in keratinocytes.

present in an inactive form in the nucleus where it becomes activated. Once actively present in the nucleus, a transcription factor binds to unique DNA sequences in the promoter region of target genes. The promoter is the DNA sequence typically adjacent to the protein-encoding DNA sequence and contains the regulatory elements that control gene transcription. Once bound to the promoter, the transcription factor activates the RNA polymerase complex, resulting in transcription of the gene into a messenger RNA, translation into a protein in the cytoplasm, and a subsequent effect on cell phenotype or behavior. Like ligands, receptors, and the steps of signal transduction, the final step of gene activation

**FIGURE 4.8**

(a) Vitamin D₃ synthesis, activation, and catabolism. (b) Ligand-activated transcription factors interact with coregulators to regulate gene transcription.

is also highly complex. Before a gene can be activated, it needs to be available for transcription in the nucleus. In contrast to the simple picture of a long stretch of helical DNA packed within the nucleus, DNA is wrapped around repeating bundles of proteins called **histones** (Fig. 4.9B). The histone proteins serve to organize the DNA in defined arrangements, where gene activation can be controlled depending on cell type and activity. For instance, embryonic stem cells will have a very different set of genes poised for activation compared to a somatic cell like a fibroblast. Without this **epigenetic** control, any gene may be activated at any time if the corresponding transcription factors are present. This could lead to inappropriate gene activation and detrimental cell functions. The epigenetic state of a cell is influenced by signal transduction through the soluble pathways outlined in Section 4.3, but also by the physical properties of the tissue environment [17], thereby making epigenetic regulation an important consideration in tissue engineering.

**FIGURE 4.9**

(a) The activation of gene transcription results in protein translation and a physiological response. (b) DNA is wrapped around repeating bundles of proteins called histones.

Outside of epigenetic control, there are additional levels of complexity in gene activation. Every gene has a unique promoter sequence and therefore recruits one or more different transcription factors. For instance, the *Runx2* gene, which is a master regulator of osteogenic differentiation, contains binding sites for the Smad transcription factors, which are activated by the BMP signal transduction cascade. Transcription, like the entire signaling cascade, does not operate as a simple on/off switch. For example, while the short Smad binding element (CAGAC) is very common in the genome and in the promoter region of genes, the presence of the sequence does not necessarily mean transcription will occur upon Smad signaling. The recruitment of Smads to their binding sites on gene promoters is determined by tissue-specific transcription factors already present in the cell [18,19]. Thus, although a majority of genes may contain potential Smad binding sites, transcriptional activation is restricted to genes appropriate to the cell type—a finding that may apply to other signaling cascades.

4.6 Variations on a theme

The paradigm of cellular signaling described in this chapter is a conceptually good starting point, but it should be noted that many signal transduction pathways use variations on this theme or use only a part of the route to convey a signal from outside the cells to evoke a biological response. First of all, some ligands directly bind and activate a transcription factor, such as vitamin D3. Moreover, the path length of the cascade differs strikingly from a single step as in BMP signaling to an elaborate cascade seen with VEGF signaling. Some signal transduction pathways exert their effect without activation of gene transcription. One of the well-established examples of this is the family of Rho kinases, which are downstream of some of the main signal transduction pathways and control cytoskeletal organization. However, more and more examples of this are beginning to emerge, again highlighting the complexity of protein interactions within cells. For example, the long cytoplasmic tail of the type II BMP receptor interacts directly with LIM kinase, thus directly affecting cytoskeletal organization [20]. Likewise, the VEGF receptor directly phosphorylates profilin, another cytoskeletal regulator. It seems inevitable that numerous examples are yet to be discovered.

Another important phenomenon in cellular signaling is **cross-talk**, in which the signals of two or more different ligands converge on the same effector molecule. Cross-talk may occur at several levels in the signaling cascade. In some cases, coactivation of a signal transduction molecule by two signaling pathways is required. For instance, CREB activation requires phosphorylation at serine-133 and serine-129, which are downstream events of GPCR signaling and insulin signaling, respectively. In other cases, the transcription factors downstream of two or even more signal transduction pathways have to bind simultaneously to a promoter in order to activate gene transcription. Finally, many signaling molecules are not uniquely activated by a single signal transduction pathway. For instance, MAPK activation can be downstream of RTKs but also of integrins. This leads to an important aspect of cellular signaling which is context—the way in which the surrounding environment can influence how a cell signals. For instance, one cellular environment may have a rich assortment of matrix molecules, while another may not. The cell in the first environment will have high integrin adhesion and downstream MAPK activity, which will be further augmented by cytokine-RTK activity. In contrast, the cell in the second environment will only have MAPK activity through the cytokine-RTK signaling. If the MAPK activity leads to gene activation, the first cell will consequently have elevated functional activity which may correspond to a particular phenotype. This example demonstrates how the properties of the surrounding environment play a major role in not only the type of signal but also its magnitude.

4.7 Future perspective

With a topic like cell signaling, it can sometimes feel that everything has been discovered. Textbooks like this one present tidy schematics of pathways from a ligand–receptor interaction through to gene expression. However, to imply that our knowledge on cellular signaling is complete is to imply that we completely understand how cells work and this could not be further from the truth. Every day, scientists are adding more to our understanding about cellular signaling. They are finding new

molecules involved in the pathways, new interactions between pathways that were previously thought to be distinct, and exception upon exception to some of the rules we thought we knew. And, importantly for tissue engineers, there are new tools being discovered and developed that can improve our chances of understanding cell signaling and controlling cell behavior. In this future perspective, some of these developments will be highlighted, and some of the gaps that still need to be filled will be revealed.

One of the most exciting developments in biology since the last edition of this textbook is the astonishing resource of molecular information that has been produced by single cell RNA sequencing. This method, which produces transcriptomic information from individual cells, has revealed new types of cells within tissues that were previously unknown, and has improved our understanding of how heterogeneous the activity of pathways can be from one cell to the next. Prior to this single cell–level information, techniques such as microarray and RNA sequencing could only tell us about an average across a population, which failed to capture important differences between cells. The reason this matters for tissue engineers is twofold. Firstly, the analyses done on these data sets have also led to the establishment of new markers. Defining a success in tissue engineering has always been a challenge, and these new markers of certain cell types help to identify whether we are supporting the right cell behavior. This is especially relevant when it comes to addressing maturity within a tissue-engineered construct, as we now have many markers that can distinguish an immature cell from one that is phenotypically complete. We can use these datasets (most of which are publicly available) to benchmark our tissue-engineered tissues to adult human tissues and get a molecular basis for any differences. Secondly, when these **transcriptomic** data are used to describe the state of signaling pathways like the ones described in this chapter, new targets emerge for tissue engineers to exploit. For example, cells found to be deficient in the activity of a given pathway could be supplemented with a ligand that activates that pathway, thereby pushing them closer to the tissue of interest. Together, the emergence of single cell RNA sequencing seems sure to improve our ability to engineer tissues and organs. As other –omics techniques such as proteomics are also moving to the single cell level, the field of tissue engineering is sure to benefit further.

Another exciting highlight has been the embrace of more complex and three-dimensional models and we are beginning to understand cellular signaling in that context. In the past, our knowledge mostly either came from model cell lines in vitro or from model organisms in development. This was always a curious challenge because the work of tissue engineers lies somewhere in between cell lines and model organisms and it was therefore always challenging to make use of the existing knowledge on pathways and their activity. Today, more and more scientists are using three-dimensional cell cultures in vitro, which provide a compromise between the simplicity of a cell line and the complexity of an organism. And most importantly, they better recapitulate the setting a cell experiences in a tissue-engineered construct. Scientists are now beginning the robust studies needed to understand cellular signaling in this setting. As more work is done in this area, tissue engineers will benefit from a deeper understanding of what governs cell behavior in their constructs.

So, what does the future hold for the topic of cellular signaling in tissue engineering? Surely it will be based on scientists and engineers developing and exploiting biologically inspired tools to influence cell behavior. This is not an insignificant challenge, and it has been an area of research since the field was first conceptualized. However, we are rapidly developing new tools such as ligands that can be incorporated into biomaterials to locally influence cells, extracellular matrix proteins or peptides mimicking their action that can be used to coat biomaterials, or growth factors, cytokines, hormones, and small molecules that can be released from scaffolds. The same compounds can also be used to control the culture medium of expanding and differentiating (stem) cells. Furthermore, materials can be designed in such a way that they elicit the desired response. Both their chemical composition and physical properties have an influence on the biological behavior of the cells growing on them. New materials for scaffolding are being developed that more accurately mimic the chemical and physical properties of native tissue, and advances in 3D biofabrication are building assemblies that are hierarchically structured from the molecular to the macroscale, thereby providing exquisite control over signaling at multiple scales. As biologists continue to unravel the complex signaling mechanisms inherent in tissue development and regeneration, more targets for tissue engineers to guide cellular assembly will become apparent.

Summary

- Most cell signaling events follow the three-step paradigm of signal initiation, signal transduction, and gene transcription.
- The presence of a ligand will not necessarily have the same or any effect on different cell types. The cellular response depends on its gene and protein expression, the extracellular environment, and many other factors.
- Cellular signaling is a complex process that is not completely understood, but there are many targets for tissue engineers to manipulate cell behavior.
- Manipulation of signal transduction for tissue engineering purposes should be considered in its in vivo context with phenomena such as cell type and cross-talk with other signaling mechanisms.
- Signaling pathways are regulated at many different levels: extracellularly, in the cytoplasm, and in the nucleus. This provides tissue engineers multiple means to manipulate the cell.

Classical experiment

A landmark study by Conboy et al. showed that mimicking Notch signaling can be used to improve muscle regeneration.

Satellite cells are the resident stem cell population that is responsible for the regeneration of muscle following injury. Like the regeneration of many tissues, the efficiency of this process diminishes with age. Indeed, when the hindlimb

muscles were injured in young (2–3 months) mice, the satellite cells produced new myoblasts and myofibers. However, in aged (23–24 months) mice, there was only 20%–25% contribution from the satellite cells and the regeneration was impaired. This could not be explained by a changing number of satellite cells, so researchers hypothesized that the dramatic decline of myoblast

Classical experiment—continued

generation in aging mice might be due to the ability of the satellite cells to be activated in response to injury.

Signaling through the Notch receptor was known to be critical for satellite cell activation. When Notch binds its ligand, Delta, which is present on adjacent cells, a protease in the plasma membrane cleaves the cytosolic tail of Notch, which then translocates to the nucleus to activate Notch target genes. Conboy et al. used fluorescence-activated cell sorting to detect the expression of Delta-1 and a Notch inhibitor, Numb, on the surface of satellite cells in their resting state and after injury. In resting cells from mice of all ages, low levels of Delta-1 and high levels of Notch were detected. In injured cells, Delta-1 was upregulated in young and adult mice, but not in old

mice, and this increase was also met by a decrease in Numb. These results were confirmed *in vivo*, where Delta expression was upregulated adjacent to the injury site in young mice but was almost unchanged in old mice. When a Notch inhibitor, Jagged-Fc, was introduced at the site of injury, the regenerative potential of young mice was inhibited.

To test whether these findings could be used to improve muscle regeneration, an antibody that binds to the extracellular domain of Notch and thereby mimics its activation was introduced at the injury site. Remarkably, by restoring Notch activation, the aged mice were able to regenerate injured muscle with the same efficiency as young mice (Fig. 4.10).

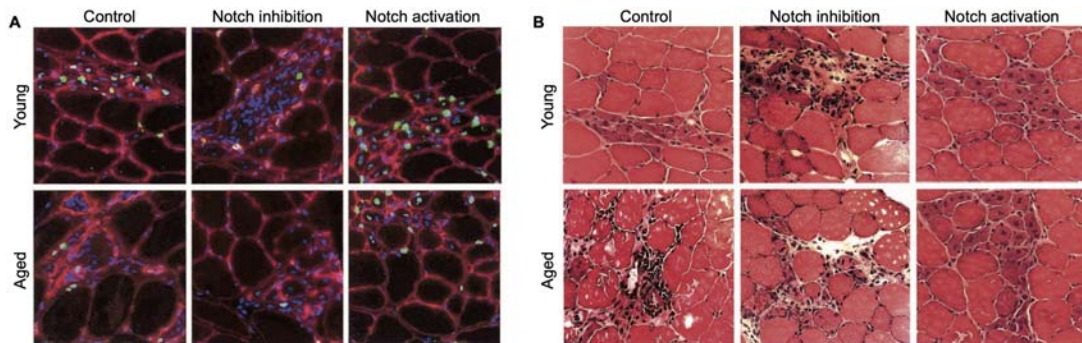


FIGURE 4.10

(a) Muscles of young and aged mice were injected with either a Notch inhibitor or activator. Immunofluorescence using antibodies against BrdU (green), showing proliferating cells, and laminin (red), marking the boundaries of myofibers, showed that Notch inhibition reduced myofiber formation, while Notch activation promoted regeneration in old muscle. (b) Hematoxylin and eosin staining confirmed the immunofluorescence results, showing the negative effect of Notch inhibition on muscle regeneration and the positive effect of Notch activation on regeneration in aged mice. *From Conboy et al. Notch-mediated restoration of regenerative potential to aged muscle. Science. 2003;302:1575–77.*

State-of-the-art experiment

Organoids mimic tissue assembly into organs at a small scale, which can help decode signaling pathways that underlie form and function. A recent study by Yu et al. demonstrated how WNT–BMP signals govern the differentiation of pluripotent stem cells and the autonomous patterning of multiple cell types to form functional “cardioids” [Yu et al., 2021].

Adult heart tissue does not fully regenerate after damage and is therefore an area of intense study in tissue engineering and regenerative medicine. During development, embryonic mesoderm undergoes multiple stages of differentiation to form the three primary cardiac cell types necessary for organ development: cardiomyocytes, endocardial cells, and epicardial cells. Human pluripotent stem cells can be coaxed to differentiate into these cell types, but without the coordinated patterning that is observed in the developing heart. Cardiac-specific patterning relies on complex spatiotemporal orchestration of signaling pathways that have not been reproduced outside of natural development. To reproduce this complexity in the laboratory, the researchers used a combinatorial screen of key cardiogenesis signal transducers including ACTIVIN, BMP, FGF, VEGF, retinoic acid, and WNT, administered to human pluripotent stem cells at key developmental stages, to identify specific conditions that facilitate cardiac development and patterning.

WNT signals during mesoderm induction led to cavity formation and impeded cardiomyogenesis in aggregates of pluripotent stem cells. In contrast, it was found that BMP signals impeded cavity formation but catalyzed cardiomyogenesis. This demonstrates how the WNT–BMP signaling axis temporally manages differentiation,

organization, and patterning during cardiomyogenesis. Screening different concentrations of signaling factors at different times revealed that low levels of both WNT and ACTIVIN during mesoderm induction are critical for in vivo–like endocardial organization in the cavity lining to mimic natural cardiac development.

After forming the endocardial lining, the next stage during heart development is the envelopment of myocardial tissue with the epicardium. Coculture with epicardial aggregates led to engraftment at the cardioid interface and differentiation to smooth muscle and fibroblast populations. Challenging these cardioids with a developmental injury model demonstrated robust response by epicardial associated fibroblasts akin to what is observed during cardiac injury. Thus, tailoring culture conditions to present WNT and BMP signals with physiologically relevant staging leads to cardioids that behave similarly to a developing heart.

This work nicely illustrates the power of manipulating cell signaling to direct functional outcomes in the laboratory. In contrast to earlier examples of engineered cardiac tissue, the exquisite control afforded through temporal administration of defined growth factors was instrumental in coaxing autonomous assembly in a way that mimics natural development. This study reveals fundamental knowledge in how signaling guides cardiac tissue assembly with scope for translating these findings for tissue engineering and regenerative medicine.

Yu et al., 2021. Cardioids reveal self-organizing principles of human cardiogenesis. *Cell* 184 (12) 3299–3317.

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4.9 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
1. List the three typical steps of cellular signaling.
 2. Describe two ways in which a ligand binding to an extracellular receptor can transmit a signal into the cytoplasm.
 3. List the three main types of ligands.
 4. Explain the biochemical properties of ligands that act extracellularly compared to those that act intracellularly.
 5. What happens to the receptor of bone morphogenetic protein 2 (BMP2) when it binds the ligand?
 6. What is the structure of a G-protein–coupled receptor?
 7. What happens to a G-protein upon binding of a ligand to a G-protein–coupled receptor?
 8. How does protein kinase A get activated?
 9. Name three members of the receptor tyrosine kinase family.
 10. Which Smads are activated by bone morphogenetic proteins (BMPs) and which are activated by transforming growth factors (TGFs)?
 11. Name the three major signaling pathways that Wnts can activate.
 12. What happens to β -catenin when Wnt binds its receptor?
 13. How does a Rho family protein get activated?
 14. Why is NF- κ B known as a latent regulatory protein?
 15. Name three roles of interleukins.
 16. Why is vitamin D considered a deviation from the cell signaling paradigm?
 17. What are transcription factors?
 18. What is a mechanism that cells use to ensure the correct set of genes is activated?
 19. How has RNA sequencing affected the field of tissue engineering?
 20. Why are three-dimensional models considered powerful for tissue engineering?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:

1. Provide an example of why you might want to promote Wnt signaling. And knowing that CHIR99021 is a Wnt activator, describe how it works.
2. Describe a scenario in which one ligand can have two different effects on cells. Provide detailed molecular information.
3. Sometimes cellular signaling proceeds by inhibiting a part of a pathway instead of activating it. Give a detailed example.
4. Describe the steps of GPCR signaling involving the parathyroid hormone–like hormone.
5. VEGF signaling contributes to the formation of new blood vessels. Draw the steps of RTK signaling involving VEGF.
6. BMP signaling contributes to bone formation. Draw the steps of TGF- β signaling involving BMP.
7. Explain how negative regulation of BMP activity occurs.
8. Draw the steps of canonical Wnt signaling.
9. How is it that some signals have very fast actions while others are comparatively slow?
10. Show and explain how different extracellular environments could lead to different levels of signal transduction in the same cell.

Challenge-based learning

Novel therapies for pulmonary fibrosis due to COVID-19 infection

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

In normal wound healing, the reparative fibrotic phase allows for the wound to be absorbed and the extracellular matrix to be remodeled to restore normal tissue function. In chronic inflammation, excessive generation and accumulation of collagen and other matrix proteins results in an increase in tissue stiffness and tissue dysfunction. It is well documented that about half of COVID-19 survivors will experience extensive lung fibrosis. The persistent respiratory complications may cause substantial population morbidity, long-term disability, and even death due to lung fibrosis progression. Therefore, the long-term goal of this research is to understand the process of lung fibrosis and generate an effective cell-based solution to resolve fibrosis after COVID-19 infection.

Motivation and stakeholders

Pulmonary fibrosis is a disease that occurs when the lung tissue becomes damaged and scarred. Most of these events are classified as interstitial lung diseases, because

it affects the tissue around the alveolar sacs. Pulmonary fibrosis may be a secondary effect of diseases like COVID-19. Autoimmune disorders, bacterial, and viral infections (as in the case with COVID-19) may cause fibrotic changes in both the lung's upper or lower lobes and microscopic injuries to the lung. Lung damage caused by pulmonary fibrosis cannot be repaired, but medications and therapies can sometimes help ease symptoms and improve quality of life. Thus, there is a clinical need to develop tissue-engineered strategies that can halt the fibrotic signaling pathway to upregulate healing mechanisms to modulate and decrease aggressive interstitial fibrotic deposition in COVID-19 patients. Solutions to mitigate this problem should consider the needs, requirements and regulatory, financial and technical boundary conditions defined by stakeholders such as patients with COVID-19, critical-care physicians, internal medicine doctors, pneumologists, cell biologist, and tissue engineers.

Problem definition

At the moment, there are no tissue-engineered approaches to stop pulmonary fibrosis and protect patients from persistent symptoms due to "long COVID." Therefore, this challenge requires the generation of a cell experiment in which the feasibility of targeting a signaling pathway that will result in the mitigation of COVID-19–induced pulmonary fibrosis is demonstrated. The experiment can either

Challenge-based learning—continued

be performed in vitro (using cultured cells), in animal models or in human patients. The motivation behind each cell and molecular strategy will have to be explained clearly, as well as the specific targets in the signaling pathway(s) to prevent interstitial lung fibrosis.

Challenge

To design and test a novel and not yet applied treatment option to resolve fibrosis in patients who developed COVID-19—induced pulmonary fibrosis.

Learning framework

Reading the Cell Signaling chapter and related literature will help you to understand the following:

1. The diseases that are associated with chronic inflammation leading to fibrosis.
2. The cell types that are associated with fibrosis in tissues and the roles they play in the disease.
3. How fibrosis leads to tissue dysfunction.
4. How fibrosis is influenced by the microenvironment of a tissue.
5. The signaling pathways that are involved in fibrosis.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

6. Genes which are activated by fibrosis related signaling pathways.
7. Shared molecular pathways between interstitial lung disease and COVID-19—induced pulmonary fibrosis.
8. The molecular cell biology of COVID-19—induced activation of myofibroblasts in lungs.
9. Current therapies to treat COVID-induced lung fibrosis.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

4.10 Glossary

Autophosphorylation is the phosphorylation of the kinase by itself to regulate its catalytic activity.

Cellular signaling is the ability of a cell to receive, process, and transmit signals with its environment and with itself.

Conformational change is a change in the shape of a macromolecule, often induced by environmental factors (e.g., temperature, pH, ionic strength).

Cross-talk refers to instances in which one or more components of one signal transduction pathway affects another.

Cyclases are enzymes that catalyze a chemical reaction to form a cyclic compound.

Cytokines are a broad category of small soluble proteins important in cell signaling.

Diffusible molecules are molecules secreted by a cell to trigger change either in itself or in other surrounding cells.

Endocytosed/Endocytosis is a cellular process in which substances are actively imported into the cell.

Epigenetics is the study of heritable phenotype changes that do not involve alterations in the DNA sequence.

Extracellular matrix (ECM) is a three-dimensional network consisting of extracellular macromolecules, such as collagen, enzymes, and glycoproteins that provide structural and biochemical support to surrounding cells.

Filopodia are present in migrating cells and are thin cytoplasmic projections that represent the continuation of lamellipodia.

Heteromeric complex formation is the formation of a protein complex that contains two or more different proteins.

Histones are proteins that bind DNA in eukaryotic cell nuclei. They act as spools around which DNA winds to create structural units called nucleosomes.

Kinase is an enzyme that catalyzes the transfer of phosphate groups from high-energy, phosphate-donating molecules to a specific substrate in a process called phosphorylation.

Lamellipodia are cytoskeletal protein actin projections on the leading edge of the cell.

Latent regulatory proteins are already present in most cells, but only activated in response to stress.

Length scale is a particular length or distance determined with the precision of one order of magnitude.

Ligand is a molecule which produces a signal by binding to a receptor on a target protein.

Lymphokines are a subset of cytokines that are produced by a type of immune cell known as a lymphocyte.

Membrane-bound proteins are proteins attached either permanently or temporally to the cell membrane.

Molecular switches are molecules that reversibly shift between two or more stable states in response to environmental stimuli.

Phosphatase is an enzyme that removes a phosphate group from a protein.

Regulated proteolysis is the specific removal or modification of proteins by proteolytic cleavage in response to specific signals.

Second messengers are intracellular signaling molecules generated in the cell in response to exposure to extracellular signaling molecules to trigger physiological changes at cellular level. Examples are calcium, IP₃, cAMP, and gases such as carbon monoxide and nitric oxide.

Signaling cascade is a series of chemical reactions that occur within a cell when initiated by an external stimulus (e.g., ligand, shear stress, toxins).

Transcription factors are proteins that control the rate of gene transcription by binding to a specific DNA sequence.

Transcriptomic(s) is the study of the transcriptome—the sum of all of its RNA transcripts, under specific circumstances or in a specific cell—using high-throughput methods, such as RNA sequencing.

Transmembrane receptors are proteins embedded in the cellular plasma membrane to act in cell signaling by conveying signals from the outside to the inside of the cell and vice versa.

Ubiquitin (Ub) is a small regulatory protein found in tissues of eukaryotic organisms. Tagging ubiquitin to a protein can mark it for degradation, alter its cellular location, affect its activity, and modify its interaction with other proteins and enzymes.

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Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Extracellular matrix as a bioscaffold for tissue engineering

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5.1 Learning objectives

After reading this chapter you will be able to:

- Understand the composition, ultrastructure, and physical properties of mammalian extracellular matrix (ECM).
- Recognize the utility of bioscaffolds derived from ECM in the field of tissue engineering.
- Understand the process by which ECM bioscaffolds are manufactured.
- Understand the meaning of “constructive tissue remodeling.”
- Identify mechanisms behind constructive tissue remodeling facilitated by ECM bioscaffolds.
- Recognize clinical applications of ECM bioscaffolds.

The whole idea about Regenerative Medicine is not to make us live forever, is quality [of life] over quantity.

S. Badylak, TEDx conference, 2019

The field of Regenerative Medicine exists because we are not very good at treating some very common and serious injuries in people.

S. Badylak, 2012

5.2 Introduction

Tissue engineering and regenerative medicine strategies for the reconstruction of injured or missing tissues have included the use of implantable scaffold materials. Such scaffolds are composed of synthetic or naturally occurring materials and are typically designed to be degradable in vivo. There are advantages and disadvantages of both synthetic and naturally occurring biomaterials. This chapter will focus solely upon biologic materials, specifically, biologic scaffold materials composed of ECM.

Cells and their environment are in constant and dynamic bidirectional communication; a process termed **dynamic reciprocity**. Through interaction with the environment, unicellular organisms assimilate information, eventually develop complex symbiotic relationships with other organisms, generate cooperative organelles, and throughout evolution, adapt complex signaling and conservation systems for immune defense, response to injury, and homeostasis to become multicellular, complex organisms.

These higher order functions evolved in multicellular systems are made possible only by organization of the extracellular space through the ECM. The ECM provides a tissue with not only structural and physical integrity, but also organizes and enables metabolism through nutrient access, determines cell fate and behavior, allows for communication within spatiotemporal constraints, and provides barriers and interfaces for intercellular and intracellular reactions. Subsystems that emerge in multicellular life including the immune system, the vascular system, and the nervous system are only made possible by the organization and function of the ECM and confer distinct functions, with various degrees of specificity and speed, required for functioning of higher order organisms. A cell divorced from dynamic reciprocity with its ECM is a cell divorced from essential functions, cooperative and competitive, required for life. In other words, the simplest single unit of a tissue is not simply the cell, but rather the cell in constant communication with its environment, the ECM.

Because of the essential role of the ECM in physiologic processes, the use of the ECM as a biologic scaffold for tissue engineering purposes has been an active area of focus for decades. The ECM represents not only a key to defining problems in health and disease through a better understanding of matrix biology and native wound healing, but also represents a potential tool: an inductive template to promote **endogenous repair** upon implantation.

ECM biologic scaffolds are manufactured by the **decellularization** of source tissues such as dermis,⁷⁰ urinary bladder,³⁶ small intestinal submucosa (SIS),^{10,80} and pericardium,⁸⁶ among others, and by the decellularization/demineralization of bone.^{33,42} The process of removing the cell from its ECM is a nontrivial task that requires a delicate balance between removal of **antigenic components** that may confer an immune rejection event in the case of **xenogeneic** ECM preparation, and preservation of the ultrastructure and biochemical milieu within the ECM. Simply stated, the process of decellularization involves removal of cells without destruction of the matrix.

Chemical, enzymatic, and/or mechanical methods are used to achieve decellularization of source tissues and such methods have marked effects upon the mechanical and biologic properties of the resultant **bioscaffold** and its potential biologic activity. Such scaffold materials have been commonly used in both preclinical and clinical applications including the reinforcement and reconstruction of musculo-tendinous structures,^{51,29} lower urinary tract,³⁵ esophageal structures,⁹ skin,⁵³ upper airway,⁴³ bone,⁴ and myocardium,⁴⁹ among others. When properly prepared, these bioscaffolds are associated with a process termed "**constructive remodeling**," which is defined as a modification of the default wound healing response, away from scarring, and toward site-specific deposition of functional tissue. The remodeling outcomes in the presence of commercially available ECM bioscaffolds have varied from excellent⁸⁰ to unsatisfactory.³⁰ The reasons for these disparate outcomes are associated with differences in decellularization and processing methods, the use of chemical cross-linking agents, and/or appropriate clinical application.

There are currently more than 80 commercially available products with a broad range of indications for use. In the past 2 decades, more than 10 million patients have been treated by surgical implantation or topical application of ECM bioscaffolds, most of which are xenogeneic (typically porcine) in origin. Because of their xenogeneic origin, early attempts to translate the use of these bioscaffolds were met with concerns: (1) the possibility of infectious disease transmission and (2) **immune-mediated rejection** events. The first of these concerns relates to animal-borne pathogens and is addressed through

maintenance of source tissue herd health and terminal sterilization of bioscaffolds. To date, documented reports of infectious disease transmission remain nonexistent, while animal-based products have been widespread in clinical use. Concerns related to immune-mediated injury stem from particular antigens not common between source animals and the recipient. Examples of potential culprit antigens include the α -Gal **epitope** and MHC-1 proteins which are contributing factors to **hyperacute rejection** responses to pig transplants in humans. The gal-epitope is expressed in nearly all mammals with the notable exceptions of Old World monkeys, humans, and apes. This concern prompted seminal studies in the early 2000s which demonstrated that though the alpha-Gal epitope is indeed present within xenogeneic ECM bioscaffolds, it exists in a very low concentration and no adverse immune responses, cellular nor humoral, are generated following implantation in primate models.²⁷ Additionally, evaluation of human blood serum following implantation of ECM bioscaffolds derived from porcine SIS did not show elevation of host antibodies.⁵ Stated differently, documented reports of infectious disease transmission or immune-mediated rejection are virtually nonexistent and are testament to the safety of these materials when they are properly decellularized and terminally sterilized. ECM bioscaffolds are typically regulated as devices by the Food and Drug Administration and therefore are subject to the same safety testing requirements as other medical devices.

Current research and biomaterial design strategies center upon the identification of the mechanisms by which the ECM, when prepared as a biomaterial, promotes functional tissue remodeling. Studies of normal wound healing, fetal development, and matrix biology intersect with transplant research and regenerative medicine to reveal substantial and crucial roles for the ECM and its constituent proteins and biochemical molecules. A hallmark of ECM-mediated tissue remodeling involves the degradation and rebuilding of the matrix itself, which occurs frequently during homeostatic matrix turnover events. Upon implantation as a bioscaffold, the ECM is rapidly infiltrated by host **polymorphonuclear** and **mononuclear cells** which initiate the scaffold degradation process. The ECM degrades relatively rapidly and completely, releasing numerous bioactive molecules that initiate a variety of cell-mediated processes. For example, **matricryptic peptides** have been shown to promote stem cell migration, proliferation, and differentiation, vascularization, and reinnervation of tissues following injury and contribute to site-appropriate tissue deposition.^{1,79} A large number of multipotent stem cells are influenced by the degradation products of ECM bioscaffolds. For example, a low molecular weight peptide derived through proteolytic degradation of the collagen III α molecule has been shown to possess osteogenic potential.³

ECM degradation products also show a robust effect on the host immune response, through both the innate and adaptive arms of the immune systems. In fact, activation of the host immune response, while initially a cause for concern due to possibility of acute or chronic rejection events, is the essential cornerstone of most beneficial responses associated with the use of these scaffolds in clinical and pre-clinical applications.²¹ While the specific components of the ECM that drive these beneficial immune responses are still an active area of investigation, recent evidence suggests that extracellular vesicles bound to the ECM, termed **matrix bound nanovesicles** (MBVs), are primary mediators of these events. Such events alter the default response of mammalian tissue to injury from the deposition of nonfunctional scar tissue to constructive, site-appropriate, and functional tissue formation (Figs. 5.1, 5.2, 5.3, 5.4, 5.5, 5.6, 5.7, 5.8, 5.9 and 5.10).

5.3 Native extracellular matrix

The ECM represents the secreted products of cells in all tissues and organs. ECM composition is generally highly conserved across tissue types; however, the three-dimensional ECM ultrastructure of each tissue is distinct. The structural and **functional molecules** that comprise the ECM provide the mechanical properties necessary for proper functioning of each tissue and facilitate signal transduction between adjacent cells and between cells and the ECM itself. The ECM fluctuates in response to changes in micro-environmental cues including cellular activity and mechanical loading, and therefore as stated above, is considered to be in a state of dynamic reciprocity with the cells and factors that influence the ECM.¹⁸

5.3.1 ECM composition

The ECM is composed of a complex combination of proteins including **structural molecules** such as collagen (Fig. 5.1), fibronectin, and laminin, as well as functional molecules such as glycosaminoglycans (GAGs) and growth factors all arranged in a tissue-specific three-dimensional ultrastructure.⁶ The individual polypeptide collagen chains consist of the repeating sequence Gly-X-Y, where X is often proline and Y is often hydroxyproline (Fig. 5.1a). Collagen is made up of three polypeptide strands which are all left-handed helices and twist together to form a right-handed coiled coil. The polypeptide strands are synthesized as precursor chains with propeptides (globular extensions) on the C and N ends. The propeptides are cleaved into short nonhelical telopeptides (Fig. 5.1b). Collagen molecules self-assemble into collagen fibrils (Fig. 5.1c). Collagen fibers are formed by end-to-end and lateral assembly of collagen fibrils, resulting in a regular banding pattern that is characteristic of collagen (Fig. 5.1d). In

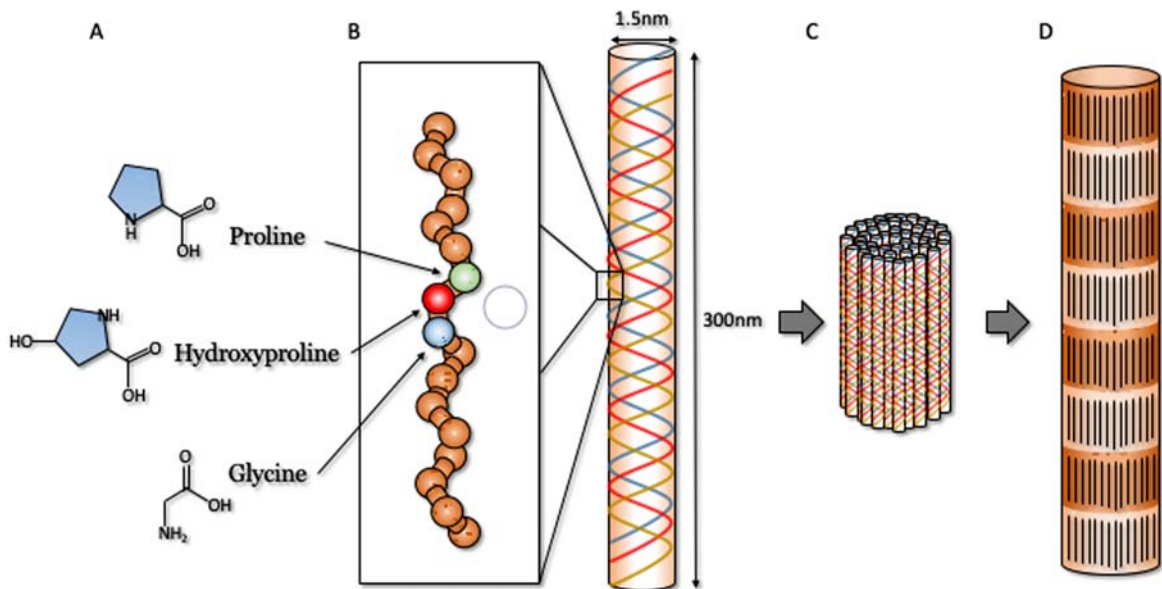


FIGURE 5.1

Schematic representation of the assembly of collagen I.

addition to molecules present within the native ECM, low molecular weight “matricryptic” peptides are liberated upon matrix proteolytic degradation and are biologically active yet distinct from native parent molecules.

Collagen

Collagen is the most abundant protein within mammalian ECM and comprises more than 90% of its dry weight.⁸³ More than 30 distinct types of collagen have been identified, each with a unique biologic function. Type I collagen is the most abundant structural protein present within tissues and is highly conserved across both animal and plant kingdoms.

Large quantities of Type I collagen are present within the ECM of tendons and ligaments, providing the necessary uniaxial and multiaxial mechanical strength required to meet the demands of these connective tissues.

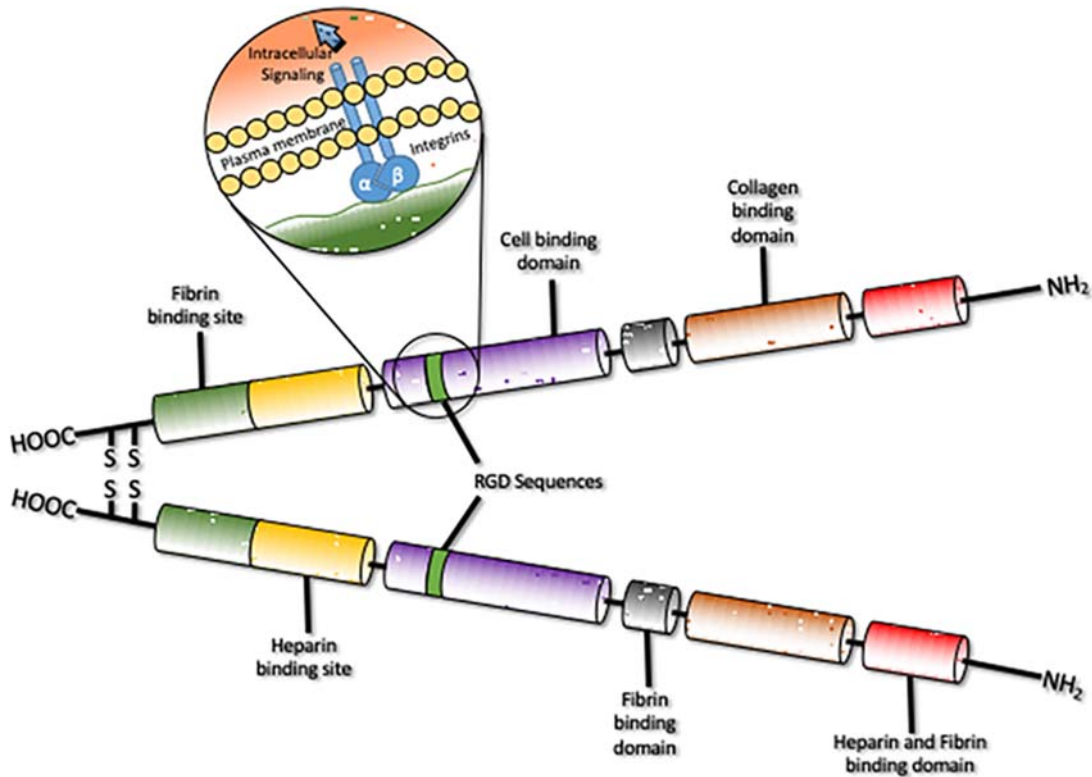
Other collagen types are present in the ECM of most tissues but typically in much lower quantities. These collagen types provide tissue-specific physical and mechanical characteristics and allow for tissue-specific cell–ECM interactions. For example, less rigid, type III collagen is present in the submucosal layers of tissues that require increased flexibility and compliance such as the urinary bladder.¹⁴ Type IV collagen has **ligand affinity** for endothelial cells and is present within the basement membrane of most vascular structures.⁸⁷ Type VII collagen is present within the basement membrane of the epidermis and facilitates fibril anchoring to protect overlying keratinocytes from shear stress.⁸⁷ Each of these types of collagen is present within most of the ECM bioscaffold materials used for constructive tissue remodeling.

Fibronectin

Fibronectin is the second most abundant ECM molecule present in both soluble and tissue isoforms in submucosal, basement membranes, and interstitial tissues (Fig. 5.2).⁴⁴ The structure of fibronectin is described as rod-like and contains three repeating modules joined together like beads on a string, called Type I, Type II, and Type III modules. Fibronectin exists in two main forms, an insoluble form found in the ECM, and a soluble form found in plasma. Rich in ligands that promote adhesion of diverse cell types including the **Arg-Gly-Asp (RGD)** subunit, fibronectin facilitates cell–ECM adhesion via the alpha5 beta1 integrin, anchoring cells to collagen or proteoglycans. Fibronectin has been shown to play a role in fetal development including regulation of cell migration and the patterning of fibronectin deposition during embryogenesis plays crucial roles in spatial arrangement and organization of organs and limb structures. Because of these characteristics, fibronectin has been used as a coating for synthetic scaffold materials to promote host biocompatibility and to control aberrant inflammation responses as well as to confer adhesion of specific cells on scaffold-cell constructs.

Laminin

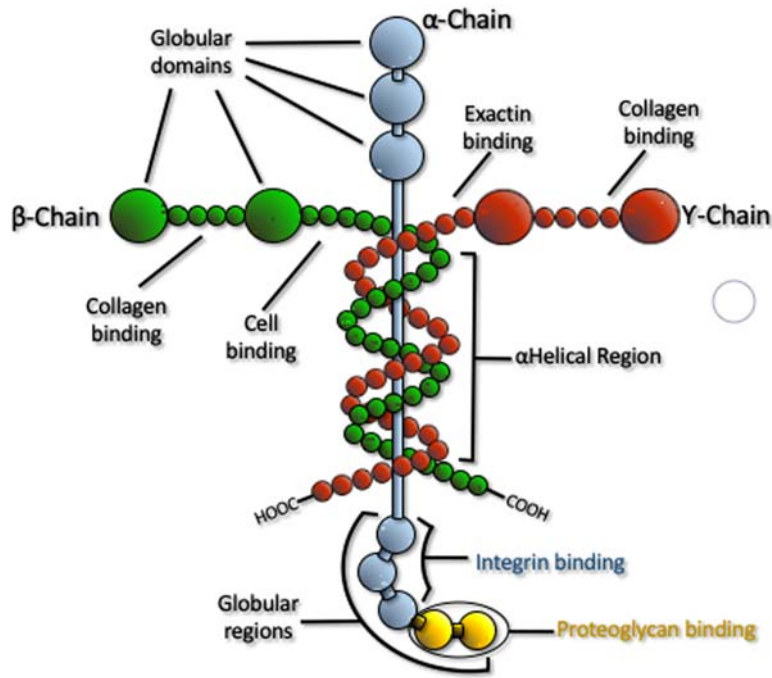
Laminin is another adhesion molecule found in the ECM, especially within basement membranes (Fig. 5.3). This trimeric cross-linked polypeptide exists in numerous forms depending upon the combination of peptide chains. Laminins are particularly known for their unusual cross-shape and are considered heterotrimers, meaning they are made of three different subunits with two short arms and one long arm. There are 16-known members of the laminin family, each associated with different

**FIGURE 5.2**

Fibronectin is a dimeric molecule joined by two disulfide bonds at the carboxyl end. Each domain of the fibronectin molecule has binding sites for cell receptors and ECM molecules.

tissue types, though they are all assembled within the cell, and are deposited into the ECM in complex patterns that are specific to cell type. These patterns have been described as “exquisite” and “remarkably specific.”⁴¹ For example, some laminins have been shown to be deposited in mesh patterns on the surface of Schwann cells and regulate their cell surface receptor and cytoskeletal organization. Lung cells deposit laminins in fibril structures and participate in mechanotransduced signaling during stretching of lung tissue. Embryonic stem cells deposit laminin in string array patterns, and keratinocytes deposit laminin in rose patterns and railroad tracks, dependent upon whether the keratinocytes are nonmigratory or migratory, respectively.⁴¹

The major role of laminins in the ECM is revealed in diseases associated with laminin mutations. Specifically, laminin is found in the basement membrane, a structure that separates parenchymal cells from connective tissue. Knockout laminin animals develop with severe disease like skin blistering, failure of organ separation in development, and kidney failure. Laminin-mediated signaling is broad, and laminins are key drivers that regulate tissue structure and organization and cellular behavior. Laminin has

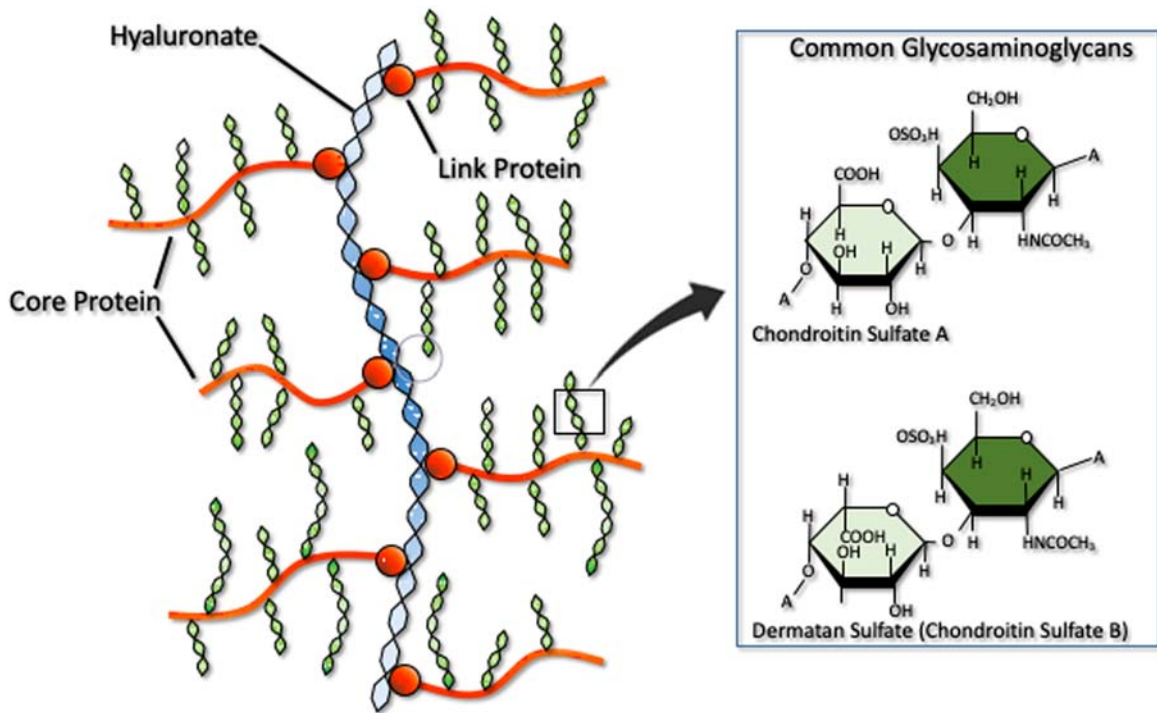
**FIGURE 5.3**

Laminin is composed of three polypeptide chains (α , β 1, and β 2) organized into the shape of a cross. Laminin has binding domains for heparin, collagen IV, heparin sulfate, and cells.

been shown to play a prominent role in the maintenance and generation of vascular structures and mesenchymal stem cell–ECM interactions.⁶⁸

Glycosaminoglycans

GAGs are long unbranched polysaccharides that are composed of a repeating disaccharide unit (Fig. 5.4). GAGs possess a variety of biologic activities including the ability to bind growth factors and chemokines/cytokines and promote water retention. The ability of GAGs to bind growth factors and cytokines allows them to form gradients of these molecules by immobilizing them on their surface. These cytokine binding properties have been demonstrated in studies that mutate GAG–cytokine adhesion sites in which the chemokines themselves remained active, but their inability to assemble within GAG binding structures results in their inability to attract cells and promote their migration. In other words, the ability of GAGs to bind growth factors and chemokines/cytokines is essential for their activity. This GAG-mediated organization and oligomer formation is found to support processes in organogenesis, immunosurveillance and inflammation, and selective cell recruitment.⁶⁹ The GAGs present in ECM include heparin, heparan sulfate, chondroitin sulfate A and B, and hyaluronic acid (HA). HA retains significant biologic activity and directly promotes cell proliferation, migration, and differentiation.⁴⁵ The concentration of HA within ECM is highest in fetal and newborn tissues and is therefore associated with enhanced healing properties. HA itself has been used in isolation as a biomaterial to

**FIGURE 5.4**

Glycosaminoglycans are long unbranched polysaccharides that are composed of a repeating disaccharide unit. The disaccharide unit is composed of one of two modified sugars, either *N*-acetylgalactosamine or *N*-acetylglucosamine.

promote wound healing and as a lubricant for arthritic joints, largely due to the ability of HA to attraction and binding of water molecules, allowing for swelling and contribution of structure and cushioning for the joint, as well as its antiinflammatory properties.

Growth factors

Intact ECM is composed of a combination of sequestered biologically active molecules present in a unique and natural spatial distribution. Although some cytokines and growth factors are present within ECM in low concentrations, they act as potent modulators of cell behavior. The list of growth factors found within ECM is extensive and includes vascular endothelial growth factor (VEGF), the fibroblast growth factor family, stromal-derived growth factor (SDF-1), epithelial cell growth factor, transforming growth factor B, keratinocyte growth factor, hepatocyte growth factor, platelet-derived growth factor, and bone morphogenetic protein (BMP), among others (Table 5.1).^{84,46,8}

Therapies delivering enriched and purified forms of growth factors, such as VEGF to promote angiogenesis or BMP to promote bone formation, have been associated with limitations including dosage and route of administration, among others. An advantage of utilizing the ECM in its intact state as a scaffold for tissue engineering is the presence of all the attendant growth factors in the same relative amounts

Table 5.1 Description of a subset of growth factors found in the ECM and their functions.

Growth factor	Acronym	Subset of function(s)
Vascular endothelial growth factor	VEGF	Stimulates formation of blood vessels
Fibroblast growth factor	FGF	Regulates cell proliferation, migration, and differentiation; plays multiple roles in wound healing and tissue homeostasis
Stromal-derived growth factor	SDF-1	Chemotactic for lymphocytes, mesenchymal stem cells, and neural progenitor cells; directs cell migration in embryogenesis
Epithelial cell growth factor	EGF	Stimulates cell growth and differentiation
Transforming growth factor B	TGF-B	Controls cell growth, proliferation, and differentiation
Keratinocyte growth factor	KGF	Promotes proliferation of epithelial cells, regulates their migration, and protects from cell death under injury or stress conditions
Hepatocyte growth factor	HGF	Regulates cell growth, migration, and morphogenesis
Platelet-derived growth factor	PDGF	Plays roles in wound healing particularly in the case of blood vessel damage; also stimulates formation of blood vessels
Bone morphogenetic protein	BMP	Induces bone formation; plays roles in homeostasis and embryogenesis

and three-dimensional ultrastructure that exist in nature. The ECM protects these growth factors from degradation and efficiently presents them to resident or migrating cells. These properties make the ECM an ideal controlled release vehicle for growth factor administration. Because the ECM is in a state of dynamic reciprocity with resident cells, these sequestered growth factors are liberated from the ECM in a controlled released manner dependent upon cellular activity in normal physiologic processes and is an area of active investigation for therapeutics which is discussed in later chapters.

5.4 ECM scaffold preparation

The preparation of an ECM scaffold derived from native mammalian tissue requires a combination of chemical and mechanical processing steps that result in efficient decellularization of the source tissue. As stated earlier, ECM scaffolds have been prepared from a variety of tissues including dermis, small intestine, urinary bladder, pericardium, liver, skeletal muscle, and adipose tissue, among others (Fig. 5.5). The analysis in this figure shows that a chemotactic fraction consisted predominantly of a single peptide with the amino acid sequence IAGVGGEKSGGF; a sequence identical to a string of amino acids form the C-terminal telopeptide of collagen III α . A BLAST search for the isolated peptide sequence showed over 75% homology with the collagen III α molecule over eight separate species.

Most source tissue materials used for derivation of ECM bioscaffolds are xenogeneic or **allogeneic** in nature, and therefore require the efficient removal of cellular antigens to prevent foreign body

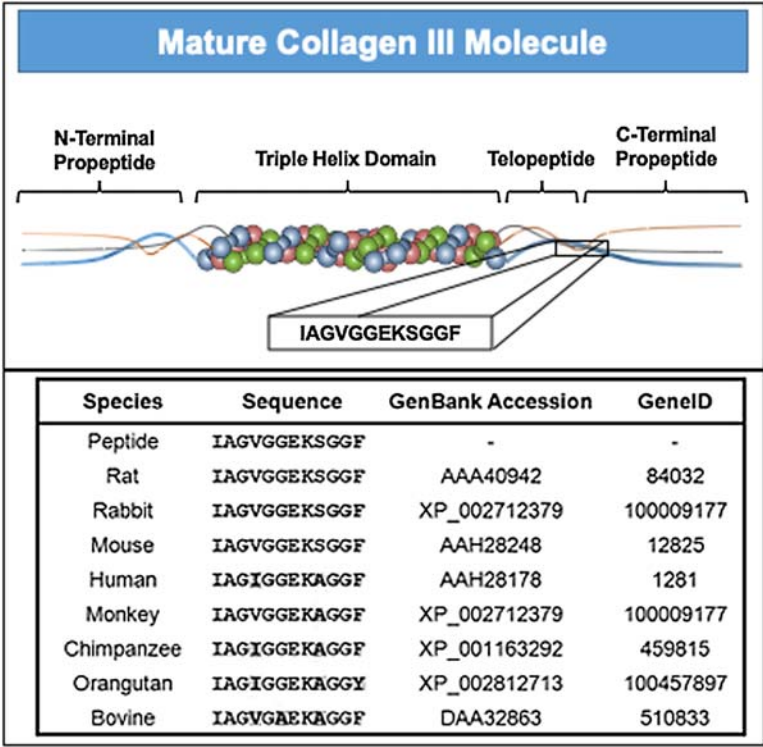
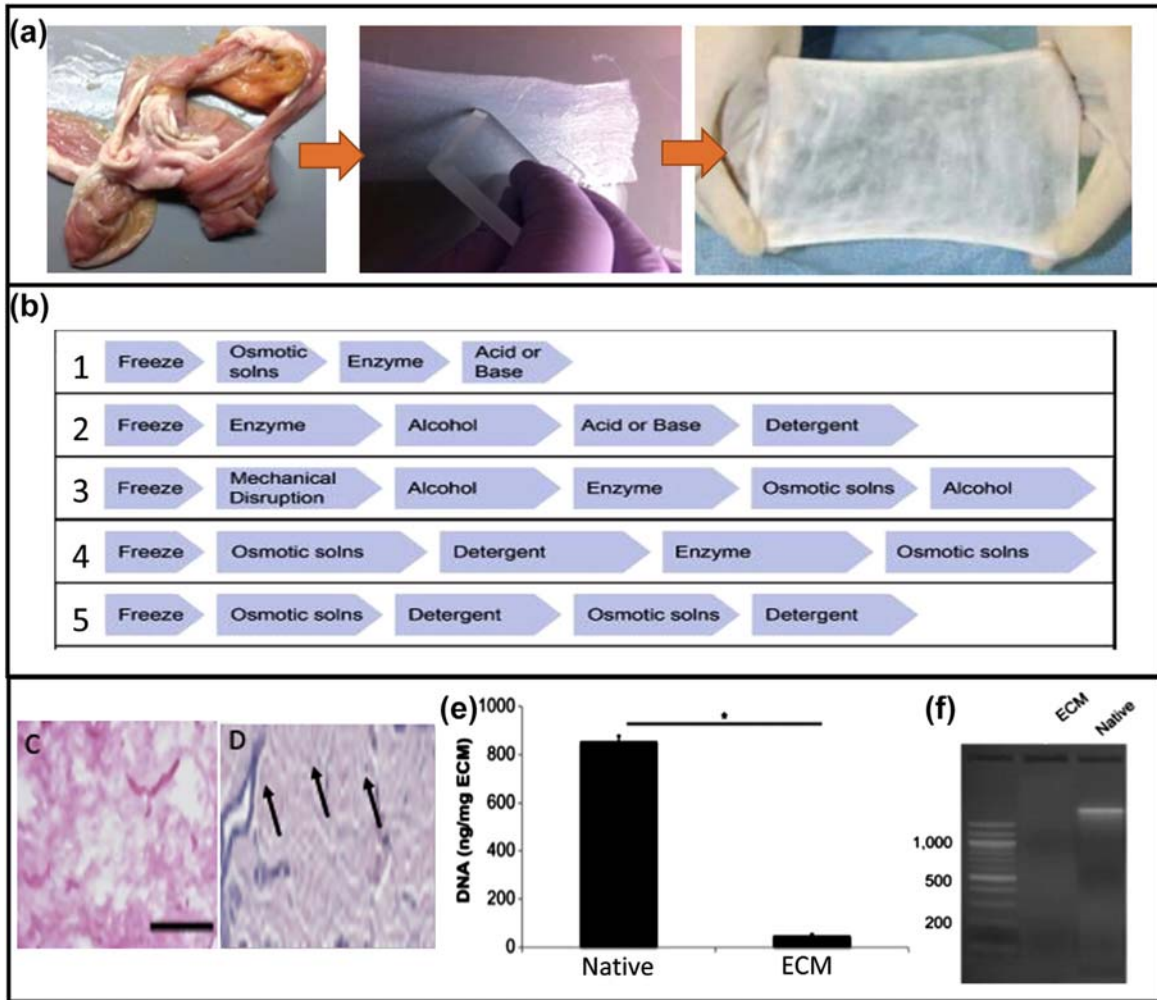


FIGURE 5.5 Peptides of urinary bladder matrix (UBM) were fractionated via ammonium sulfate precipitation and subsequent size exclusion, ion exchange, and reverse phase chromatography.

recognition by the host following in vivo implantation. Depending upon the tissue of interest, mechanical methods such as manual or automated **delamination** of muscle and **mucosal layers** are typically followed by a combination of physical, chemical, and enzymatic methods to achieve complete decellularization. The goal of any decellularization process is the removal of all cellular and nuclear material while maintaining the composition, microstructure, mechanical properties, and biologic activity of the remaining native ECM. A biologic scaffold material is considered to be efficiently decellularized when it meets the following criteria: (1) lack of nuclei present within the scaffold material following histologic or chemical nuclear staining; (2) total scaffold material dsDNA content must be less than 50 ng/mg; (3) any remaining DNA remnants present in the scaffold material must be less than 200 base pairs in nucleotide length (Fig. 5.6).²⁴ An example of a properly decellularized biologic scaffold is the urinary bladder matrix (UBM). The UBM is mechanically decellularized by scarping and chemically decellularized by peracetic acid. The resultant acellular material is white in appearance (Fig. 5.6a). Examples of decellularization protocols for (1) thin laminates such as pericardium, (2) thicker laminates such as dermis, (3) fatty, amorphous tissues such as adipose tissue, (4) composite tissues or whole simple organs such as trachea, and (5) whole vital organs such as liver have been developed. Arrow lengths represent

**FIGURE 5.6**

The urinary bladder matrix as an effectively decellularized biologic scaffold. *Adapted from An overview of tissue and whole organ decellularization processes, Crapo PM, Gilbert TW, Badyalak SF. An overview of tissue and whole organ decellularization processes. Biomaterials 2011;32:3233–3243.*

relative exposure times for each processing step. The criteria for efficient scaffold material decellularization suggest the following (Fig. 5.6b): the decellularized material should nuclei after H&E histology (Fig. 5.6c) compared to native tissue (Fig. 5.6d, arrows indicate positive nuclei staining) and should lack 4,6-diamidino-2-phenylindole (DAPI) staining in fluorescent images (Fig. 5.6e). The material should contain less than 50 ng/mg dsDNA dry weight. Remaining DNA fragments should be less than 200 bp in length (Fig. 5.6f).

Recent studies also suggest that other components of remnant cells including cell membranes and mitochondria can also play a role in determining the host response to ECM biomaterials.⁵⁴ Physical treatments for decellularization include **sonication**, scraping and/or shaking, or subsection to freeze–thaw cycles. Such methods facilitate the disruption of cell membranes and removal of cell contents from the native ECM. Enzymatic treatments with detergents or ionic solutions further denature cell membranes and disrupt intercellular bonds. Because the ECM of different tissues varies with regard to composition, structure, and density, the combination of methods used to achieve efficient decellularization varies across tissues.

Following decellularization, ECM intended for use as a bioscaffold must be sterilized prior to surgical placement. Sterilization of biologic materials, including ECM bioscaffolds, involves unique considerations such as the shrink temperature of collagen, potential cross-linkage of bioactive components, and potential changes in surface characteristics, among others. Despite these considerations, numerous methods for the terminal sterilization of ECM scaffold materials have been identified. These techniques include ionizing radiation such as gamma and electron beam irradiation, and exposure to ethylene oxide (ETO) gas, among others. The challenge of terminal sterilization of ECM bioscaffolds is efficient reduction of **bioburden** without destruction of the structural and functional molecules that comprise the ECM. For example, a comparison of ETO, electron beam, and gamma irradiation showed that increasing radiation dosage has a detrimental effect on the mechanical properties, i.e., **tensile strength** and degradation rate, of ECM bioscaffolds, whereas ETO sterilization results in a reduction of growth factor content.²⁸ Alternative terminal sterilization methods have been evaluated, particularly for ECM hydrogels, to maintain structure and function. One such method includes the use of supercritical CO₂ which has been shown to be an effective sterilization approach for preservation of ECM hydrogel stiffness and gelation time. Optimization of ECM sterilization, particularly of hydrogels, is an area of ongoing investigation in biomaterial design and development.

5.5 Constructive tissue remodeling

5.5.1 Default mammalian wound healing versus constructive remodeling

The default mammalian response to injury is highly conserved across tissue types and occurs in three overlapping yet distinct phases: the inflammatory phase, the **proliferative phase**, and the **remodeling phase**.⁷⁶ The **inflammatory phase** occurs immediately after tissue damage and is marked by activation of the coagulation cascade to promote **hemostasis** and by the subsequent influx of **innate inflammatory cells** that facilitate microbicidal activity and the removal of cellular debris. Hemostasis is achieved through the deposition of a provisional fibrin matrix which acts as a physical scaffold for infiltrating neutrophils and macrophages.²³ The proliferative phase is characterized by angiogenesis and the migration, proliferation, and differentiation of various cell types.⁶⁶ During the remodeling phase, excess macrophages and endothelial cells undergo apoptosis, while fibroblasts and myofibroblasts secrete a collagenous ECM. This host-derived fibrous tissue matrix represents the precursor of scar tissue, which replaces site-appropriate functional tissue within the injury site.

In contrast, constructive tissue remodeling is a process by which the default healing response following injury is modified from one that culminates in scar tissue formation to one that results in the formation of site-appropriate functional tissue. Mechanisms behind an ECM scaffold-mediated constructive

remodeling response to injury are discussed in the following section and include the participation of an activated host progenitor cell population and a predominant M2/Th2 immune response among others (Fig. 5.7).^{11,15} Efficient decellularization removes proinflammatory cellular material and is therefore necessary for a constructive tissue remodeling response. Conversely, cross-linking prevents scaffold degradation and as a result decreases the release of beneficial matricryptic peptides and growth factors within the ECM. Poor decellularization, bacterial contamination, and/or cross-linking may induce a host response consistent with a **foreign body reaction** and results in scar tissue formation (blue staining; collagen) and some adipose deposition (arrows) (Fig. 5.7a). Thoroughly decellularized and sterilized, noncross-linked scaffolds promote site-appropriate tissue remodeling (arrows: skeletal muscle fibers) (Fig. 5.7b). The scaffold remodeling process occurs through a series of events following scaffold implantation (Fig. 5.7c).

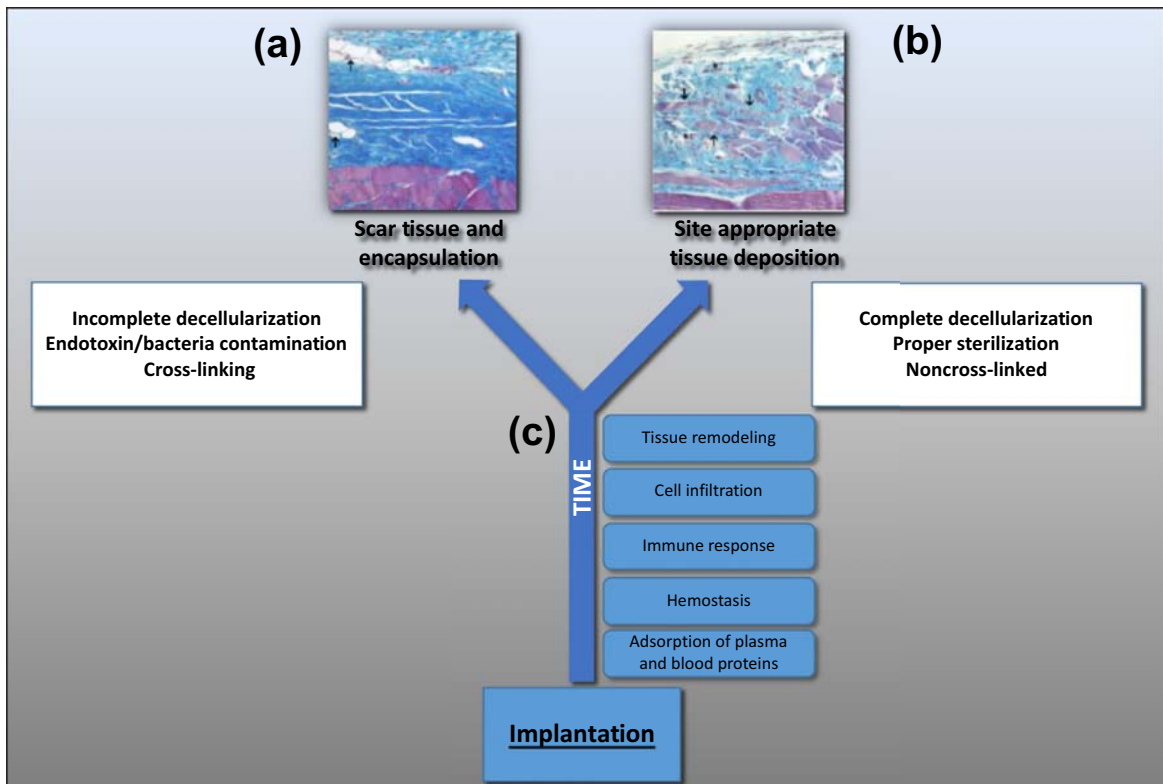


FIGURE 5.7

The remodeling outcome associated with biologic scaffolds depends upon a variety of factors including the effectiveness of the scaffold decellularization process,⁴⁷ and whether or not the scaffold material has been chemically cross-linked,⁸¹ among others.

5.5.2 Mechanisms behind ECM-mediated constructive tissue remodeling

While the specific mechanisms behind the ability of ECM-based bioscaffolds to promote constructive tissue remodeling remain only partially understood, there are a number of processes which are believed to be key contributors. Among these processes are: 1) the ability of the scaffold material to degrade rapidly with concomitant release of bioactive signaling molecules, 2) the recruitment and activation of stem/progenitor cells, and perhaps most importantly, 3) the modulation of the innate immune response. Macrophages, in particular, are key facilitators of ECM bioscaffold degradation and the subsequent regulation of inflammation, fibrosis, and stem/progenitor cell activation, proliferation, and differentiation.^{22,82} Each of these processes is discussed in the following section and explored in further detail in the studies which comprise this chapter.

Scaffold degradation

Recent studies suggest an important role for ECM-derived biologically active molecules, including growth factors, matricryptic peptides, and a variety of cytokines and chemokines. The release of ECM-derived signaling molecules is dependent upon ECM degradation. Therefore, when used as a surgically placed biologic scaffold material, ECM degradation is a necessary event for constructive tissue remodeling. Quantitative studies of ¹⁴C-labeled ECM bioscaffolds show that approximately 50% of the ECM scaffold is degraded within 28 days and virtually all of the bioscaffold is replaced by host tissue by 60–90 days postimplantation,⁴⁰ a process partially dependent upon the anatomic site of placement. In misguided attempts to strengthen the mechanical properties of ECM bioscaffolds, **chemical cross-linking** with chemicals like glutaraldehyde or carbodiimide has been used. However, the use of chemical cross-linkers typically results in undesirable outcomes and a lack of host tissue integration. Chemical cross-linked ECM bioscaffolds prevent macrophage-mediated scaffold degradation and initiate a foreign body response through **frustrated phagocytosis**, persistent inflammation, the presence of multinucleate giant cells, and ultimately scar tissue formation that encapsulates the bioscaffold with dense fibrous tissue.⁸² Stated differently, adverse outcomes tend to occur with the use of ECM bioscaffolds and chemical cross-linking agents.

Endogenous cell therapy by ECM bioscaffolds

Degradation of the ECM by physical or chemical methods creates low molecular weight peptides that promote a variety of biologic events including chemoattraction for stem and progenitor cells. These ECM-derived peptides range in size from 5 to 16 kDa and have demonstrated chemotactic activity for primary endothelial cells,⁵² perivascular stem cells (PVSCs),²⁶ and neural progenitor cells,²⁵ among others.

The constructive remodeling properties of ECM bioscaffolds extend beyond the integration of the ECM with surrounding host tissue. For example, primitive pluripotent stem cells which express Sox2⁺, a transcription factor, were shown to be present at the site of ECM implantation in an adult mammalian model of digit injury.^{1,2} Multipotent bone marrow-derived cells have also been shown to participate in the remodeling of ECM scaffold-mediated Achilles tendon repair.⁸⁸ Specifically, in a preclinical Achilles tendon injury model, ECM scaffold explants promoted significantly increased chemotaxis of progenitor cells after 3, 7, and 14 days of in vivo remodeling compared to an autologous graft control.¹⁵ The results of the study showed greater migration of progenitor cells toward tendons repaired with ECM

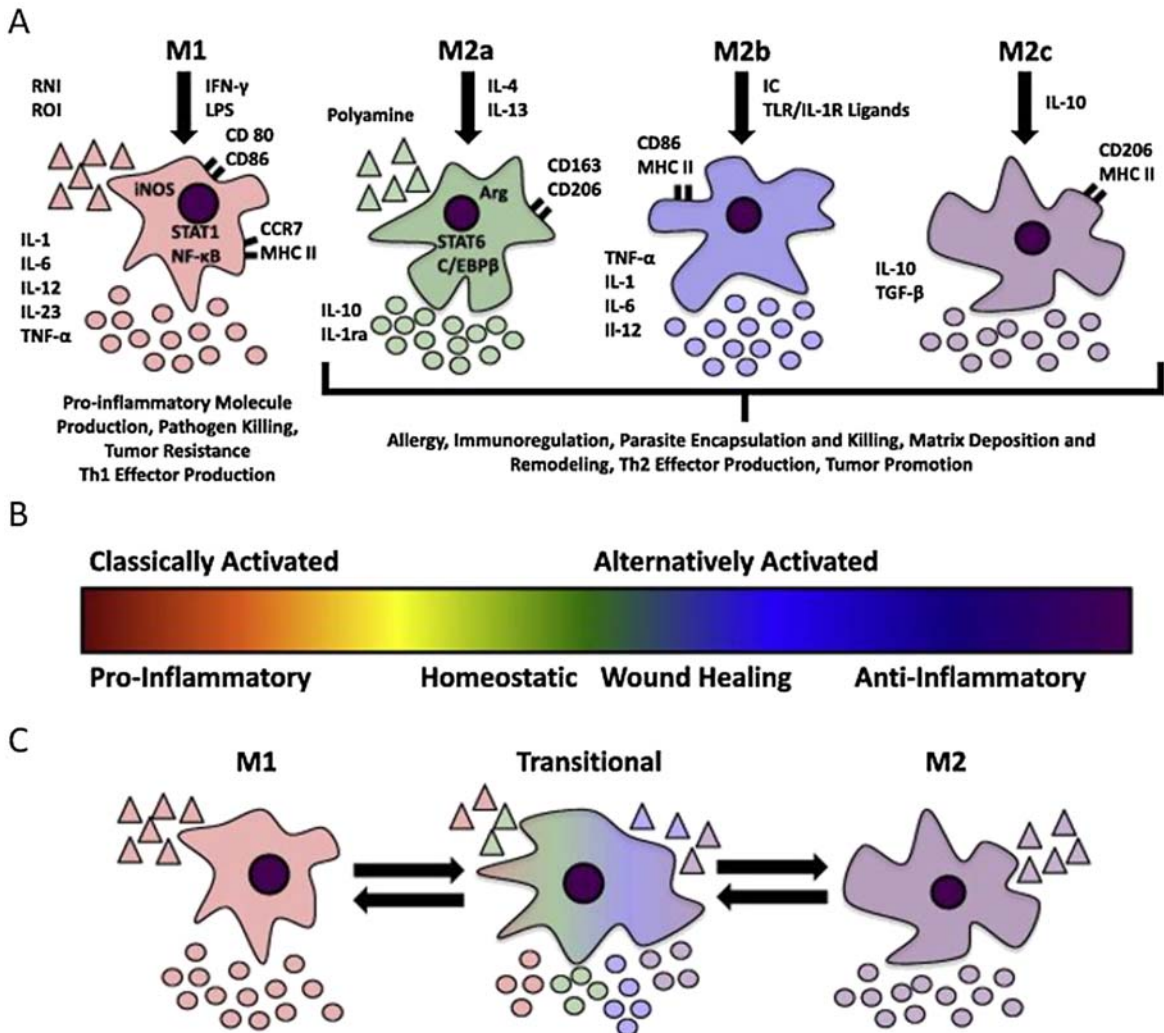
scaffolds compared to tendons repaired with autologous tissue and uninjured normal tendon. ECM bioscaffolds have also been associated with robust reinnervation of skeletal muscle tissue, an important factor in preventing denervation atrophy.¹⁵ ECM bioscaffold placement within a volumetric muscle loss (VML) injury model showed presence of beta-3 tubulin positive nerve cells within the center of the remodeling site and subsequent muscle remodeling associated with improved functional outcomes.^{31,32} These results support the concept that ECM scaffolds are capable of recruiting progenitor cells to the site of implantation to participate in constructive tissue remodeling over the long term.

Modulation of the host immune response by ECM bioscaffolds

The host innate immune system and especially macrophages play a pivotal role in the host response to implanted biomaterials, including those composed of metals,⁷⁷ ceramics,⁵⁵ polymers,⁵⁰ and biologic proteins such as collagens and xenogeneic ECM.⁸² **Foreign body giant cell** formation and proinflammatory cytokine production are frequently associated with the implantation of nondegradable or synthetic biomaterials (Fig. 5.8). It is now accepted that macrophages are capable of diverse phenotypes and also function as regulators of tissue homeostasis during the host response to disease and tissue injury. Among these processes are the ability of the scaffold material to degrade rapidly with concomitant release of bioactive signaling molecules, the recruitment and activation of stem/progenitor cells, and perhaps, most importantly, the modulation of the innate immune response can be seen in (Fig. 5.8a). Macrophage polarization occurs along a spectrum between M1 and M2 extremes (Fig. 5.8b). Macrophage phenotype is plastic and can change in response to paracrine and autocrine signals (Fig. 5.8c). Triangles in this figure represent secreted reactive species and circles represent secreted cytokines. In addition to this, several recent studies suggest that immunomodulatory macrophages can facilitate constructive and site-appropriate tissue remodeling in response to ECM bioscaffold implantation.^{82,75}

Macrophage heterogeneity

Until approximately the year 2000, macrophages were considered antimicrobial phagocytes and mediators of chronic inflammation and fibrosis.^{48,72} Macrophages are part of the innate immune system, but studies during the past few decades have revealed a much more complex and heterogenous role for macrophages. These cells are now recognized as a cell type with remarkable plasticity and are essential for normal fetal development, healthy tissue homeostasis, and a spectrum of tissue responses following injury that range from fibrosis/scar tissue to functional tissue regeneration.^{20,56,59,61} Macrophages have been categorized according to their functional properties as either M1 or M2.⁵⁸ **M1-like macrophages** are proinflammatory or “classically activated” cells stimulated by proinflammatory factors (such as IFN γ and LPS). M1-activated macrophages secrete toxic reactive oxygen species, nitric oxide intermediates, and inflammatory cytokines such as IL-1 β , IL-6, and TNF- α . These cells are responsible for microbicidal activity, debris clearance, and the propagation of a proinflammatory and **Th1 response**. Conversely, “alternatively activated” and constructive **M2-like macrophages** are stimulated by immunomodulatory and antiinflammatory factors such as IL-4 and IL-10. M2-activated macrophages express IL-10, have high levels of scavenger and mannose receptors (i.e., receptors associated with phagocytosis), and produce arginase in the place of inducible nitric oxide synthase, subsequently producing ornithine and polyamines. M2-like macrophages are responsible for propagation of a **Th2 response**, matrix deposition, and tissue repair and reconstruction.⁵⁷

**FIGURE 5.8**

Macrophages have typically been recognized as phagocytic and antigen-presenting cells responsible for propagating the proinflammatory response of the mammalian innate immune system. *From Expanded applications, shifting paradigms and an improved understanding of host-biomaterial interactions, Brown BN, Badylak SF. Expanded applications, shifting paradigms and an improved understanding of host-biomaterial interactions. Acta Biomater 2013;9:4948–4955.*

Most relevant to this chapter is the phenomenon of surgically placed ECM bioscaffolds to be associated with a spatiotemporally regulated switch from an M1-like to an M2-like macrophage phenotype. The phenotype of responding macrophages has been found to be an important determinant factor in the success of an implanted biomaterial scaffold and its ultimate remodeling outcome.^{11,21} Recent work suggests that the inflammatory component of many diseases or chronic injuries results from aberrant

immune switching, not only in macrophages but also in neutrophils, eosinophils, and T cells. Resolution of inflammation and modulation to reparative phenotypes through therapeutic intervention is an area of active investigation in both the biomaterials and pharmaceutical fields.

ECM bioscaffolds promote a constructive macrophage phenotype

ECM bioscaffolds promote a transition from a predominantly M1 macrophage cell population shortly following surgical placement to a predominant M2 cell population by 7–14 days postimplantation.^{11,21} As stated earlier, the phenotype of host macrophages which respond to biologic scaffold materials upon implantation has been shown to serve as a statistical predictor of the resultant tissue remodeling outcome. Synthetic scaffold materials (e.g., polypropylene mesh) as well as chemically cross-linked ECM scaffold materials, which do not degrade and are therefore unable to liberate bioactive signaling molecules, are associated with a robust and prolonged M1 macrophage phenotype and ultimately result in a chronic inflammatory response. Conversely, noncross-linked ECM bioscaffolds, capable of degradation and liberation of embedded signaling molecules, are associated with an early transition from an M1 phenotype to a predominant M2 macrophage phenotype and result in constructive remodeling including the deposition of site-appropriate and functional tissue. These results suggest that interactions of host cells with degradation products from ECM bioscaffolds may affect macrophage phenotype. While the exact bioactive component(s) of the matrix responsible for this phenomenon remains unknown, a better understanding of macrophage–scaffold interaction has potential to affect future biomaterial design and application.

Antimicrobial properties of ECM bioscaffolds

Naturally occurring antimicrobial peptides are commonplace within both the plant and animal kingdoms. It has been shown that components of ECM scaffolds contain antibacterial activity against both **gram-negative** and **gram-positive bacteria**.⁷¹ Furthermore, this antibacterial activity is conserved across several tissue types. Specifically, degradation products from ECM scaffold materials derived from the small intestine, bladder, and liver showed bactericidal activity and that degradation of the scaffold material is necessary for the inhibition of bacterial growth.¹⁹

Scaffolds composed of ECM are resistant to bacterial infections in both preclinical and clinical applications.¹³ In one preclinical study, dogs were subjected to segmental aortic replacement with either a synthetic graft or with an ECM bioscaffold, both of which were deliberately contaminated with *Staphylococcus aureus* at the time of implantation. Dogs implanted with the synthetic graft had positive bacterial cultures and showed the presence of persistent infection after 30 days. However, dogs implanted with ECM had negative bacterial cultures and were free of an adverse inflammatory response after 30 days.⁷

The antibacterial effects of ECM degradation may prevent immediate postimplantation infection before the host innate immune system becomes activated. Additionally, as the ECM continues to degrade over time, the continued release of bioactive molecules may provide sustained antibacterial effects.

5.6 Clinical translation of ECM bioscaffolds

ECM bioscaffolds have been surgically implanted in more than 10 million patients during the past 2 decades. ECM-based bioscaffolds are decellularized, disinfected, and terminally sterilized according

to strict QC and GMP standards and are most commonly implemented to reinforce soft tissue by promoting tissue ingrowth and constructive remodeling. The ability of an ECM bioscaffold to promote meaningful clinical results is perhaps best demonstrated in notoriously difficult-to-treat injuries and diseases. The remainder of this section discusses the use of ECM bioscaffolds to promote healing in clinical applications such as skeletal muscle reconstruction and esophageal preservation, both of which have limited treatment options, high morbidity, and typically poor outcomes.

5.6.1 Skeletal muscle reconstruction

Adult mammalian skeletal muscle tissue retains remarkable regenerative capacity following minor trauma such as exercise-induced injury. Specialized skeletal muscle progenitor cells called **satellite cells** are responsible for this regenerative plasticity. Following injury, signals from the injured skeletal muscle microenvironment stimulate resident satellite cells to proliferate and give rise to myoblasts, which go on to fuse and form contractile skeletal muscle myofibers. In the case of minor injury, these regenerated myofibers are able to functionally replace the injured skeletal muscle tissue.

When traumatic soft tissue injuries result in a massive loss of skeletal muscle tissue, the resulting defect overwhelms the regenerative response of skeletal muscle. Such devastating trauma, referred to as volumetric muscle loss (VML), is commonplace on modern battlefields and results in significant cosmetic and functional deficits for wounded soldiers. Limited treatment options for VML, including autologous tissue transfer, are associated with complications such as **donor site morbidity** and failure of graft integration. The constructive tissue remodeling associated with surgically placed ECM bioscaffolds suggests a potential therapeutic application for VML treatment (Fig. 5.9). The VML was a result of injuries sustained in battle. The injuries were treated with surgical placement of a biologic scaffold composed of porcine SIS ECM. CT scan showed the presence of radiopaque tissue formation (arrows), consistent with skeletal muscle, at the site of ECM bioscaffold implantation, at 4 months postsurgery.

In vitro studies showed degradation products from ECM scaffold materials were **chemotactic** for myogenic skeletal muscle myoblasts and PVSCs.^{85,26} In a mouse model, surgical placement of an ECM bioscaffold within sites of VML promoted the formation of islands of skeletal muscle cells after 56 days. Furthermore, in a preclinical canine model, an ECM bioscaffold remodeled into contractile skeletal muscle tissue after 6 months when placed within a VML defect affecting gastrocnemius skeletal muscle and associated Achilles tendon musculotendinous tissue.

A recent clinical case study highlighted the ability of an acellular ECM scaffold to promote skeletal muscle constructive remodeling within a wounded soldier suffering from VML of the quadriceps femoris muscle.⁶⁰ At 16 weeks postimplantation, the patient showed a 30% increase in muscle function that was concomitant with the presence of soft tissue consistent with skeletal muscle on **computed tomography (CT) scan**.

Furthermore, a clinical cohort study which evaluated the effect of ECM implantation at the site of VML in 13 patients showed the successful use of ECM biologic scaffolds to promote muscle repair and improve patient quality of life.^{31,32} The outcome measures of this trial include mechanical strength and function; the cell populations involved in the process, quality of life in these patients, as well as to examine the cellular composition of the remodeled tissue. Results showed significant strength (average of 25% increase)

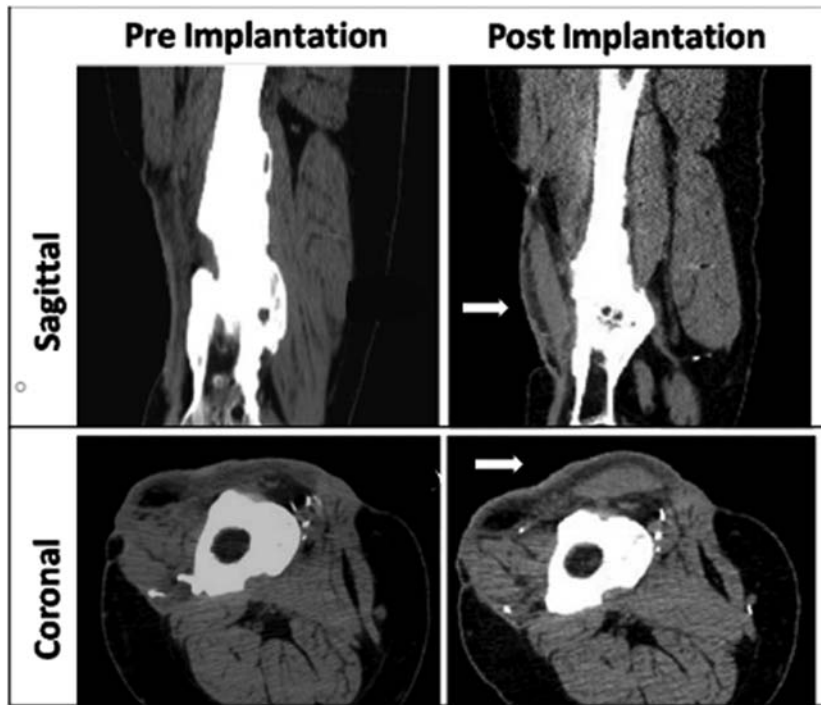


FIGURE 5.9

A marine (military personnel) suffering from VML of the quadriceps femoris muscle. Adapted from Mase VJ Jr, Hsu JR, Wolf SE, et al. *Clinical application of an acellular biologic scaffold for surgical repair of a large, traumatic quadriceps femoris muscle defect*. Orthopedics 2010; 33:511.

and functional improvements (average of 200% increase) in all compared to their presurgical maximum performance, evidence of reinnervation following nerve conduction studies, presence of new muscle tissue, mobilized PVSCs, and nerve cells from biopsy immunolabeling, and bulk muscle tissue deposition shown by MRI and CT scans. This study showed that though the patients had variable sites of injury and had all previously exhausted their standard of care treatment options (i.e., grafting surgeries and physical therapy), ECM bioscaffold placement combined with physical therapy significantly improved their quality of life 24 weeks following implantation. Taken together, these results demonstrate the potential clinical efficacy of ECM scaffolds in promoting the recruitment of endogenous stem cells and facilitating restoration of structure and function in volumetric skeletal muscle defects.

5.6.2 Esophageal mucosa reconstruction

The default response to injury in the **esophagus** caused by chronic gastroesophageal reflux disease (GERD), **iatrogenic injury**, or ingestion of caustic agents, among other causes, is characterized by inflammation resulting and rapid, robust scar tissue deposition and stricture. Use of the ECM to mitigate these responses demonstrates the potent antifibrotic, proremodeling responses triggered by the ECM.

The use of the ECM in clinical cancer scenarios addresses a potential concern: namely, are they safe in a cancerous environment which is characterized by aberrant tumor growth? When patients present with esophageal cancer (esophageal adenocarcinoma [EAC]) or precancerous lesions in the esophagus (Barrett's esophagus), the stage of cancer will dictate treatment options. Specifically, when confined to the inner lining of the esophagus, the mucosa, the lesions can be resected or ablated. However, the extent of resection is limited by the length of the lesion, since as the length of resected mucosa increases (i.e., above 6 cm in length), the risk of complications including bleeding, perforation, and stricture formation. Recent advancements in therapies including stepwise radiofrequency ablation and radical endoscopic resection require numerous interventions and can lead to high incidence of metachronous lesions.⁷³ As a result, the standard of care for many esophageal diseases, especially overt stage 1 cancer confined to the mucosa and its precursor Barrett's disease with **high-grade dysplasia**, involves watchful waiting and eventually performing radical esophagectomy, removal of the entire esophagus, a procedure associated with high complication rates that approach or exceed 50%.⁶⁷

The severity and disappointing treatment options for esophageal disease show the remarkable potential of a regenerative medicine/tissue engineering–based therapy. An ECM bioscaffold–based approach has been promising as a viable treatment for esophageal disease in both preclinical and clinical studies (Fig. 5.10). Upper Row: Left, Complete circumferential, 8 cm long, mucosectomy and inversion exposed the underlying esophageal muscularis externa. Middle, Stent deployment facilitated placement

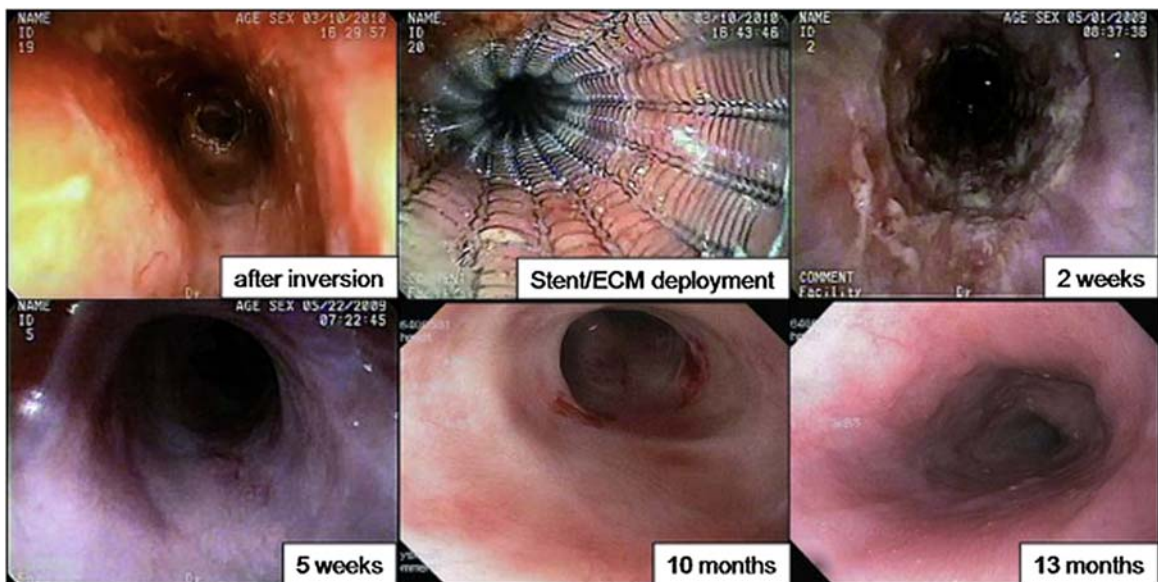


FIGURE 5.10

Representative endoscopic images of ECM bioscaffold placement in a patient suffering from superficial esophageal adenocarcinoma. *From Esophageal preservation in five male patients after endoscopic inner-layer circumferential resection in the setting of superficial cancer: a regenerative medicine approach with a biologic scaffold, Badyak SF, Hoppo T, Nieponice A, Gilbert TW, Davison JM, Jobe BA. Esophageal preservation in five male patients after endoscopic inner-layer circumferential resection in the setting of superficial cancer: a regenerative medicine approach with a biologic scaffold. Tissue Eng 2011;17:1643–1650.*

and gentle compression of a tubularized ECM bioscaffold against the muscularis externa. Right, Endoscopy at 2 weeks, immediately after stent removal, showed the ECM scaffold material firmly placed against the area of resection. Bottom Row: Left, the resected area showed the presence of squamous epithelium and no ECM scaffold material was detectable after 5 weeks. Middle, the esophageal mucosa appeared normal with short segment circumferential strictures after 10 months. Right, the resected area was covered by normal esophageal epithelium without stricture formation at 13 months.

To date, 14 patients with EAC have been treated with an ECM-based approach. The entire mucosa following resection is replaced with an ECM bioscaffold, held in place by a stent. This aggressive resection strategy, left without ECM placement, results in **stricture formation** within weeks in all cases. However, surgical placement of an ECM bioscaffold can significantly modify the scarring response,⁶⁵ even in a highly-prone scarring scenario like the full resection of a mucosa layer. Results from preclinical studies laid the foundation for the successful clinical translation of this regenerative medicine approach in these patients. These patients were nonsurgical candidates for esophagectomy as a result of **comorbidities**. Placement of an ECM bioscaffold induced the formation of a neomucosa free of disease⁹ and significant stricture complications. This regenerative medicine approach has a potential to change the practice of medicine for patients affected with EAC, the cancer type showing the greatest rate of increase in recent years.³⁴

Recent work has also shown the utility of other formulations of ECM biomaterials to make a clinical impact. A preclinical Barrett's esophagus model treated with oral administration of an ECM hydrogel showed complete reversion of the disease within 30 days of treatment compared to treatment with proton pump inhibitors alone.⁶³ Further preclinical work using a rat model of EAC suggests that this approach may even be effective in more severe progression.⁹⁰ This innovative work suggests that patients with severe GERD, which typically would develop into precancerous or cancerous disease in a subset of patients, can be targeted earlier in a noninvasive manner. In other words, by conditioning the cells with a normal microenvironment in the form of the ECM, the cells will revert to their normal phenotype. These studies and others demonstrate remarkable promise for designing improved tissue engineering therapies that focus on restoration of not just normal cell behavior, but restoration of a normal, healthy environment to drive therapeutic success.

5.7 Commercially available scaffolds composed of ECM

Scaffold materials composed of ECM are currently being used in several tissue engineering applications. In addition to the clinical studies discussed earlier, ECM scaffolds are being used to treat chronic dermal wounds such as nonhealing diabetic ulcers, and for the reconstruction and reinforcement of abdominal wall, pelvic floor, and orthopedic musculotendinous tissue (Table 5.2).

5.8 Future perspective

Tissue engineering and regenerative medicine strategies include the use of cell-based therapies, scaffold-based approaches, and/or the use of bioactive molecules. Optimal approaches will undoubtedly differ for each clinical application. However, it is clear that ECM-based bioscaffolds have a prominent place in the regenerative medicine armamentarium of therapeutic applications. Successful tissue engineering

Table 5.2 Commercially available products composed of intact extracellular matrix.

Product	Manufacturer	Source tissue	Subset of outcomes
AlloDerm	Lifecell	Human skin	5 year follow-up shows no safety concerns and host tissue integration. ³⁹
AlloPatch	Musculoskeletal Transplant Foundation	Human fascia lata	
Axis dermis	Mentor	Human dermis	No associated adverse events and promotes timely closure of diabetic foot ulcers. ⁸⁹
Bard* Dermal Allograft	Bard	Cadaveric human dermis	
CopiOs*	Zimmer Inc.	Bovine pericardium	Early studies suggest minimal adverse events and promotion of neovalvular tissue deposition; some complications occur in high-pressure intracardiac sites. ⁶⁴
CorMatrix ECM	CorMatrix Cardiovascular	Porcine small intestine	
CuffPatch	Arthrotek	Porcine small intestinal submucosa (SIS)	Favorable safety and efficacy profile for dural repair. ¹⁶
DurADAPT	Pegasus Biologicals	Horse pericardium	
Dura-Guard	Synovis Surgical	Bovine pericardium	Safe and effective for rotator cuff repair. ⁷⁴
Durasis	Cook SIS	Porcine small intestinal submucosa (SIS)	
Durepair*	TEI Biosciences	Fetal bovine skin	Safe and effective for diabetic foot ulcer treatment. ³⁸
Epic	St. Jude Medical Inc.	Porcine heart valve	
FasLata	Bard	Cadaveric fascia lata	Significantly improves wound healing of chronic leg ulcers over compression therapy alone. ⁶²
FortaFlex	Organogenesis Inc.	Porcine small intestine	
Freestyle	Medtronic Inc.	Porcine heart valve	
Graft Jacket	Wright Medical Tech.	Human skin	
Hancock II	Medtronic Inc.	Porcine heart valve	
IOPatch	IOP Inc.	Human pericardium	
Matristem	Acell	Porcine urinary bladder	
Meso	Kensey Nash Corp.	Porcine mesothelium	
Biomatrix	Medtronic Inc.	Porcine heart valve	
Mosaic	Mentor Worldwide LLC	Human dermis	
NeoForm	Healthpoint	Porcine small intestinal submucosa (SIS)	
Oasis	Pegasus Biologicals	Horse pericardium	
OrthADAPT	Bard	Porcine dermis	
Pelvicol	Synovis Surgical	Bovine pericardium	
Peri-Guard	Tissue Science Laboratories	Porcine skin	
Permacol	TEI Biosciences	Fetal bovine skin	
PriMatrix	Edwards Lifesciences LLC	Porcine heart valve	
Prima Plus	DePuy	Porcine small intestinal submucosa (SIS)	
Restore			

Table 5.2 Commercially available products composed of intact extracellular matrix. *Continued*

Product	Manufacturer	Source tissue	Subset of outcomes
SJM Biocor	St. Jude Medical Inc.	Porcine heart valve	Safe and effective for inguinal hernia repair with comparable morbidity to synthetic meshes. ¹⁷
Stratasis	Cook SIS	Porcine small intestinal submucosa (SIS)	
Strattice	Lifecell Corp.	Porcine dermis	
SurgiMend	TEI Biosciences	Fetal bovine skin	
Surgisis	Cook SIS	Porcine small intestinal submucosa (SIS)	
TissueMend	TEI Biosciences	Fetal bovine skin	
Veritas	Synovis Surgical	Bovine pericardium	
Xelna	Molnlycke	ECM protein, PGA, water	
Xenform	TEI Biosciences	Fetal bovine skin	
Zimmer Collagen Patch	Tissue Science Laboratories	Porcine dermis	

and regenerative medicine therapies will require cognizance of the simplest unit of a functional tissue: the cell in dynamic reciprocity with its ECM, and not the cell alone. The complexity of the ECM is only just being realized. Projects like the Matrisome Project attempt to develop a robust inventory of the molecules contained with the ECM. Matrix biology and wound healing research is constantly revealing the roles of the ECM in nearly all physiologic processes of multicellular life, beginning with embryogenesis, orchestrating homeostasis and wound healing, driving or resolving disease pathogenesis, and even contributing to aging processes. The recent use of three-dimensional scaffolds composed of whole organs, which have been decellularized, represents a potential major advance for patients with end stage organ failure.¹² Novel approaches to develop ECM-based biomaterials include injectable hydrogels, matrix bound nanovesicles (MBVs), and isolates of soluble and structural components of the matrix. The development of coatings and ECM-mimicking materials have begun to pave the way toward development of satisfactory treatments for unmet clinical needs. Moreover, an understanding of the role of the native ECM, the milieu within, and how its structure and functions change spatiotemporally and in response to stimuli is undoubtedly required to develop innovative approaches.

In summary, by taking advantage of Mother Nature's ideal scaffold, ECM, tissue engineering, and regenerative medicine approaches can have an immediate impact upon the practice of medicine. Reverse engineering the environment of cells is key to unlocking the potential of the ECM as a biomaterial and holds promise for development of future therapeutics.

Summary

- Scaffolds composed of ECM are useful in many tissue engineering applications.
- The ECM is composed of structural and functional molecules arranged in a tissue-specific three-dimensional structure.
- The main components of the ECM are collagen, fibronectin, laminin, and GAGs.
- Following isolation from various tissues, the ECM maintains structural and functional proteins that support constructive tissue remodeling.
- Constructive tissue remodeling is distinct from the default scar forming injury response and results in the formation of site-appropriate and functional tissue.
- ECM bioscaffold-mediated constructive tissue remodeling is dependent upon scaffold degradation.
- ECM bioscaffolds are associated with endogenous cell therapy including the recruitment of progenitor cells and modulation of the innate immune response. Specifically, ECM scaffolds are associated with a constructive M2 macrophage phenotype.
- ECM bioscaffolds promote constructive remodeling of soft tissues and have been translated from preclinical models to clinical implementation in patients suffering from VML and EAC.

Classical experiment

An assay for the identification of chemotaxis induced by degradation products from ECM bioscaffolds.

Tissue engineering strategies have utilized the transplantation of exogenous stem/progenitor cells, referred to as cell therapy, for the treatment of injured or missing tissues. These strategies have been associated with limitations including immunologic rejection of the cells and failure of the cells to incorporate within host tissue. ECM scaffold materials have been shown to release cryptic bioactive molecules upon their degradation *in vivo*. These matricryptic peptides recruit endogenous host stem/progenitor cells to sites of ECM bioscaffold surgical placement. The implementation of acellular bioscaffolds composed of ECM may obviate the limitations associated with exogenously delivered cell therapy by recruiting

endogenous host-derived stem/progenitor cells to sites of tissue injury. The study described here uses a chemotaxis chamber assay to examine the ability of degradation products from ECM bioscaffolds to recruit PVSCs.

Biologic scaffolds composed of porcine UBM were digested as previously reported,³⁷ resulting in the creation of bioactive degradation products including matricryptic peptides. The potential chemotaxis of PVSCs toward degradation products of UBM was examined using a chemotaxis chamber assay as previously described.¹ Briefly, UBM digests were placed in the bottom wells of a chemotaxis chamber, while PVSCs were placed in the top. A membrane containing 8 μm sized pores, which trapped migrating cells, separated UBM from cells. Cells were allowed to migrate for 3 h at 37°C. At the end of the

Classical experiment—continued

experiment, nonmigrated cells are washed from the membrane, while migrated cells are counted, and chemotaxis is quantified. The assay showed that degradation products

from biologic scaffolds composed of UBM are chemotactic for multipotent PVSCs and that low oxygen conditions potentiated this effect [Fig. 5.11].

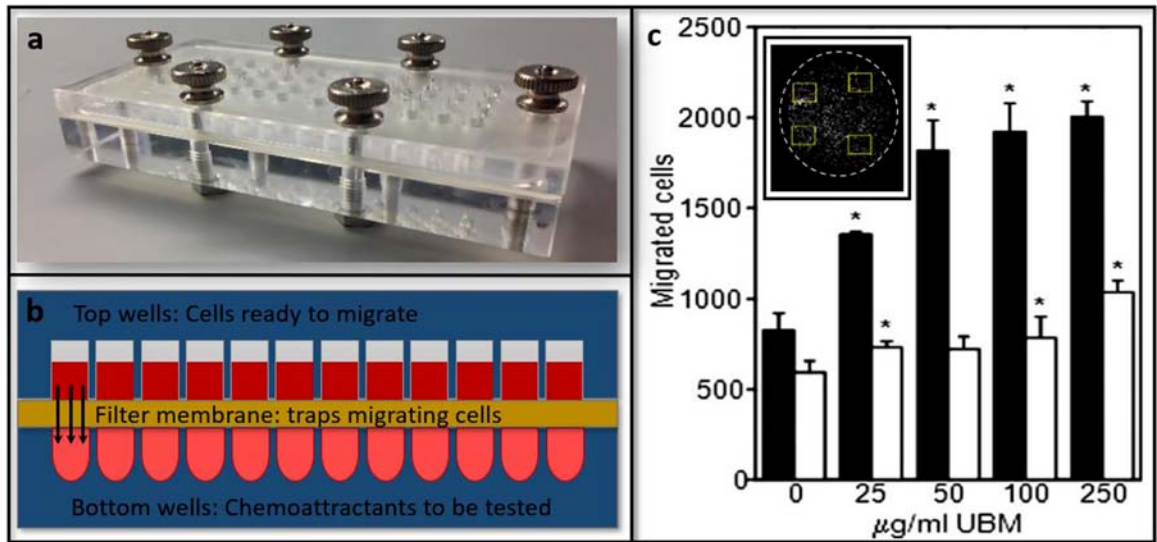


FIGURE 5.11

(a) The present study utilized a chemotaxis chamber. (b) PVSCs were placed in the top wells, while degradation products from UBM were placed in the bottom wells. (c-insert) Migrated cells were trapped in an 8 µm pore membrane and stained with a nuclear stain for counting and subsequent quantification. (c) The present study showed that degradation products from UBM promoted the chemotaxis of PVSCs in a dose-dependent manner (white bars), and that low oxygen conditions (black bars) potentiated this effect. *From Extracellular matrix degradation products and low-oxygen conditions enhance the regenerative potential of perivascular stem cells. Tissue Eng 2011;17:37–44. Agrawal V, et al. Epimorphic regeneration approach to tissue replacement in adult mammals. Proc Natl Acad Sci USA 2010;107:3351–3355.*

State-of-the-art experiment

ECM bioscaffolds have been shown to promote positive results to prevent stricture following resection of cancerous lesions in the esophagus. Though long-term (10 years) follow-up of these patients has not shown any cancer recurrence, concern that the ECM's ability to promote a proregenerative microenvironment would exacerbate cancer cell growth remains without targeted investigation of the effect of healthy ECM upon cancer cell phenotype. The present study examines the effects of ECM hydrogels from healthy tissue upon healthy versus cancerous cell activity. In other words, this experiment poses the question: if provided with a healthy (i.e., nonmalignant) environment, will cancerous cells operate in dynamic reciprocity and change their phenotype toward a healthy phenotype?

To evaluate the isolated effect of the environment on cell behavior, an *in vitro* model of dynamic reciprocity was used. Normal (Het-1A), metaplastic (CP-A), and neoplastic (SKGT-4, OE33) esophageal epithelial cells were exposed to ECM hydrogels prepared from healthy porcine tissue. Outcome measures that were evaluated included metabolic activity of the cells using an MTT assay, apoptosis (programmed cell death) using flow cytometry to evaluate Annexin V/Propidium iodide (PI) expression, proliferation using a BrdU assay, and activation or inactivation of known cancer signaling pathways like the PI3K-Akt-mTOR

pathway and cell cycle/DNA replication signaling and autophagy using western blotting and qPCR.

The results of this study showed that ECM prepared from healthy tissues increased apoptosis and robustly downregulated PI3K-Akt-mTOR and cell cycle/DNA replication signaling in of metaplastic and neoplastic cell lines and upregulated the autophagy signaling pathways in neoplastic cells while decreasing their proliferation. However, exposing healthy ECM to healthy cells increased their proliferation and cell cycle/DNA replication signaling. Results of this study emphasize the power of dynamic reciprocity: provision of a healthy environment to a malignant cell can trigger the cell to behave more like a healthy cell and less like a malignant cell. The pathways evaluated in this study are also implicated in EAC progression, demonstrating significant clinical implications.

These results can be corroborated in preclinical models of EAC using the Levrat model, a validated surgical preclinical model of EAC in Sprague–Dawley rats in which an end-to-side esophagojejunal anastomosis is created; thereby, constant duodenal and gastric reflux enters the esophagus. Over approximately 6 months, animals will develop an EAC phenotype that can be confirmed by histology and monitored by health indicators like weight loss and continual endoscopic monitoring. This model can

State-of-the-art experiment—continued

corroborate in vitro findings of the potency of ECM hydrogels in an environment of neoplastic disease and gastric reflux without lesion resection. Using this model, the efficacy and deliverability of ECM-based therapeutics like an

ECM hydrogel as a therapeutic to promote cancer regression and remission can be evaluated in an in vivo setting, underscoring the clinical utility of ECM-based biomaterials (Fig. 5.12).

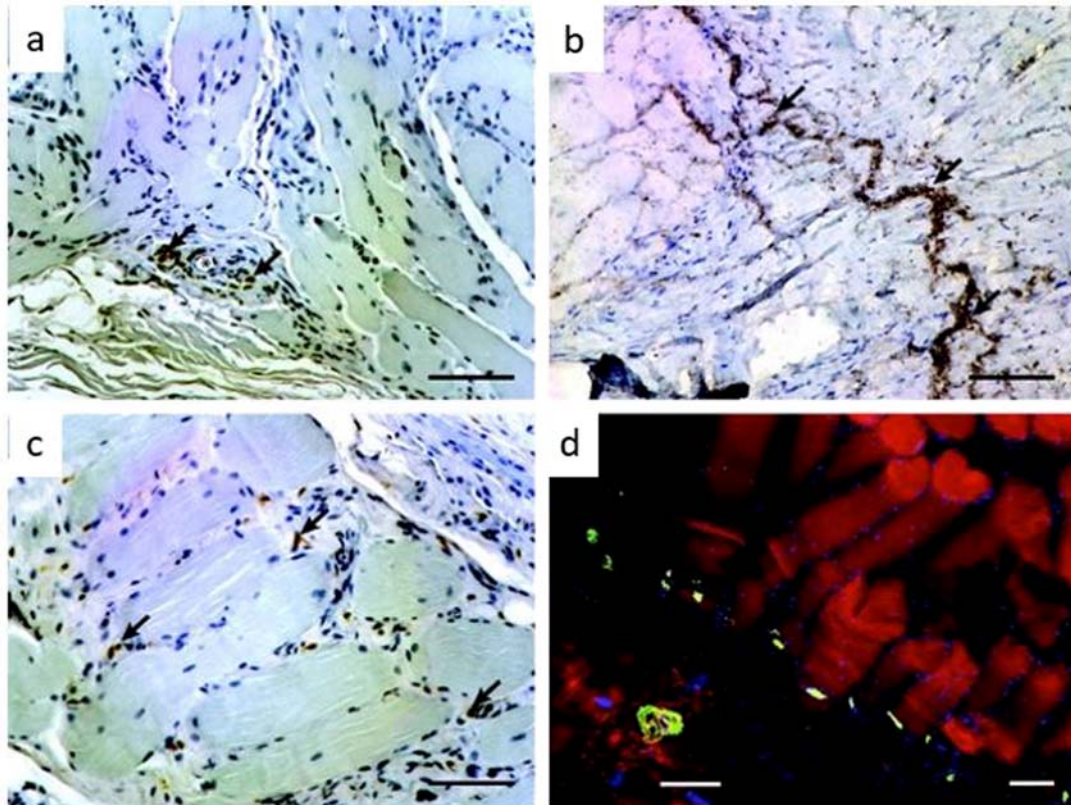


FIGURE 5.12

Innervation of remodeled small intestinal submucosa-ECM implants. Sections (a–c) were immunolabeled for β -tubulin 3 to identify neurons (brown) within the remodeled tissue. In the fluorescent image (d), α -bungarotoxin (green) showed many motor end plates within regenerated skeletal muscle tissue (red). From *Xenogeneic extracellular matrix as an inductive scaffold for regeneration of a functioning musculotendinous junction*. Tissue Eng 2010;16:3309–3317. Saldin LT, et al. *Extracellular matrix degradation products downregulate neoplastic esophageal cell phenotype*. Tissue Eng Part A 2019;25:5–6.

5.9 Recommended literature

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5.10 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
 1. Name the main structural components of the extracellular matrix and one function of each.
 2. Define the phrase “constructive remodeling.”
 3. What is the term used to describe the removal of cellular components from the ECM to produce a biologic scaffold?
 4. What are the important factors to consider in preparing an ECM-based scaffold?
 5. What other forms of ECM-based biomaterials have been produced besides surgical meshes?
 6. What host cell type has been implicated in predicting constructive remodeling upon ECM bioscaffold implantation?
 7. List the main contributing mechanisms to the ECM-mediated constructive remodeling response in chronological order.
 8. Mention the three criteria used to determine the adequate decellularization of an ECM mesh.
 9. Give three examples of ECM biomaterials used in a clinically translated context.
 10. What is a foreign body reaction?
 11. What is the general structure of a collagen molecule?

12. Describe techniques used to chemically cross-link ECM bioscaffolds.
 13. Xenogeneic ECM sources are often used in tissue engineering applications. What is one major concern associated with the use of xenogeneic materials?
 14. Which structural ECM component is responsible for binding growth factors and contributing to water retention?
 15. How many types of collagen have been identified?
 16. Name two common glycosaminoglycans.
 17. Describe the main structural components of integrins.
 18. Name three important roles of the native extracellular matrix in physiology.
 19. What is the difference between an M1 versus an M2 macrophage?
 20. Explain why, technically speaking, the word “inert” biomaterial is misleading.
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:
1. Describe the overall manufacturing process of an ECM bioscaffold.
 2. Describe the role in the host response to ECM bioscaffolds in perpetuating a constructive remodeling outcome.
 3. Utilizing ECM bioscaffolds has been described as an acellular tissue engineering approach. How, without supplemented cells, can ECM function as a tissue engineering scaffold?
 4. What is the general difference in host response and final outcome associated with synthetic, nonresorbable versus biologic resorbable scaffold materials?
 5. Which processing factors have been found to contribute to negative outcomes associated with the use of ECM scaffolds?
 6. Describe the Boyden Chamber assay. What is it used for? What are the primary parameters that are manipulated in the assay?
 7. What is meant by the description of macrophages as a “plastic” cell type?
 8. How does “constructive remodeling” differ from tissue regeneration?
 9. What is the rationale for the use of ECM from xenogeneic sources to promote tissue repair?
 10. What is meant by “dynamic reciprocity” and how is it relevant to the design and application of ECM as a biomaterial?

Challenge-based learning

Modification and engineering of extracellular matrix enzymes to reverse and resolve fibrosis

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

The extracellular matrix (ECM) is a ubiquitous and dynamic network of macromolecules that forms a scaffold for cells, tissues, and organs. The ECM is organized in a tissue-specific manner and is constantly undergoing remodeling due to its interaction with proteins, growth

factors, and other bioactive molecules. Thus, the ECM can continuously change the microenvironment the resident cells are exposed to. These ECM changes can alter processes like homeostasis, cellular proliferation, differentiation, tissue development, cellular adhesion, and migration. In addition, the ECM can guide the body's response to injury. Abnormal response and/or chronic tissue injury results in excessive accumulation of ECM in and around damaged organs. This causes scarring or fibrosis and, if unchecked or uncontrolled, can lead to organ failure. A long-term goal of the healthcare industry is to develop therapeutic methods to prevent or control organ fibrosis.

Challenge-based learning—continued

Motivation and stakeholders

Fibrotic diseases of organs (kidneys, lungs, liver, and heart) remain a major health problem globally and account for one third of all deaths worldwide. Even though organ transplantation is currently used to manage organ failure, reasons such as shortage of organs, complications due to life-long immunosuppression in transplant patients, and transplant rejection make this option less than ideal. The study of the fibrosis process reveals that it shares common but complex cellular and molecular pathways across organs. Fibrosis is also neither static nor irreversible since matrix-degrading enzymes produced by the ECM play an important role in tissue remodeling and wound healing. Targeting these enzymes by regulating their expression can be an efficient tool for limiting or reversing fibrosis. Solutions to mitigate organ fibrosis should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as patients suffering from fibrotic diseases, the clinicians involved in their care, biomaterial scientists, and bioengineers.

Problem definition

At the moment, there are no approved treatments that use targeted administration of matrix-degrading enzymes to control fibrosis and associated organ failure. There is a need for a strategy where an off-the-shelf implant carrying matrix-degrading enzymes could be activated at the fibrosis site.

Challenge

To design a biomaterial-mediated strategy by delivering engineered enzymes to increase their matrix-degrading activity to target organ fibrosis without sacrificing the tissue's normal structure and function.

Learning framework

Reading the Extracellular Matrix chapter and related literature will help you to understand that:

1. ECM is highly heterogenous and the composition varies between tissues. Revise and understand the basic components and organization of the ECM that are commonly encountered in all tissues.
2. Fibrosis results in inflammation caused by a variety of stimuli, which leads to excessive deposition of

ECM components. Describe what triggers fibrosis and what steps are involved in this process.

3. Describe the molecular mechanisms that trigger fibrosis which are shared across tissues and organs.
4. Understand the pathologic signaling pathways involved in fibrosis.
5. Define tissue regeneration and how it is different to fibrosis.
6. Describe the ECM factors and pathways responsible of tissue regeneration after injury.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

1. The triggers for the resolution of fibrotic tissue. Include the function of fibroblasts, myofibroblasts, and fiber-degrading enzymes.
2. Study the delivery modes to locally restrict the activity of matrix-degrading enzymes. Include strategies to control the dosage of these enzymes at the injury site.
3. Understand the different strategies being used by tissue engineers to slow down or disrupt fibrosis to regain organ function.
4. Describe the common targets in the ECM pathway that can be used to reverse fibrosis.
5. Describe and understand the different types of implants (natural and synthetic) being used to reverse fibrosis. Also, study the potential response the hosts can triggered upon implantation of such materials.
6. Understand the mechanism of action of matrix-degrading enzymes involved in fibrosis.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

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5.11 Glossary

- Allogeneic** are cells, tissues, or organs derived from antigenically dissimilar individuals from the same species.
- Antigenic components** are molecules that can trigger an immune response in an immunocompetent organism.
- Arginine-Glycine-Aspartic Acid (Arg-Gly-Asp RGD) subunit** is a tripeptide motif present in extracellular matrix proteins that serves as an attachment site for cells to the extracellular matrix.
- Bioburden** is defined as the number of microorganisms on/in a biomaterial before sterilization.
- Bioscaffold** is a structure of natural origin used as a support to repair and/or regenerate a tissue or organ structure.
- Chemical cross-linking** refers to a covalent bond between two molecules caused by a chemical agent.
- Chemotactic** is unidirectional movement of an organism or entity triggered by a chemical gradient.
- Comorbidities** are the presence of one or more disease conditions that exist in addition to a primary disease.
- Computed tomography (CT) scan** is a noninvasive medical imaging technique in radiology used for diagnostic purposes to produce three-dimensional images of the body.
- Constructive remodeling** is a modification of the default wound healing response, different from scarring, and toward site-specific deposition of functional tissue.
- Decellularization** is a technique by which a tissue or organ is processed by various physical, chemical, and/or enzymatic methods to remove all the resident cells leaving behind only the extracellular matrix which later will serve as a bioscaffold.
- Delamination** is the separation of layers in a multilaminate structure
- Donor site morbidity** is the presence of discomfort and/or reduced function at the site of tissue harvest.
- Dynamic reciprocity** is a continuous, two-way interaction between cells and their immediate microenvironment, for instance, between cells and the surrounding extracellular matrix.
- Endogenous repair** is the process of healing by cells and signaling molecules that exist within the individual.
- Epitope** is part of the antigen that is recognized by the immune system and is bound by the antibody.
- Esophagus** is a muscular tube, approximately 25 cm long in the human, that connects the throat (pharynx) with the stomach.
- Extracellular matrix (ECM)** is a three-dimensional network consisting of extracellular macromolecules, such as collagen, enzymes, and glycoproteins, that provide structural and biochemical support to surrounding cells.
- Foreign body giant cell (FBGC)** is a multinucleate cell resulting from the fusion of several macrophages.
- Foreign body reaction (FBR)** is the host tissue response to the presence of a (typically nondegradable) foreign material. The classic FBR includes multinucleate giant cells, a persistent mononuclear cell response, and a fibrous capsule around the foreign material.
- Frustrated phagocytosis** is the process by which phagocytic cells, typically macrophages, attempt to phagocytose an object but are unable to do so because of its large size.
- Functional molecules** are macromolecules having a specific biologic activity or task in an organism.
- Gram-negative bacteria** are bacteria that possess a thin layer of peptidoglycan between two membranes (diderm). They give a negative result in the Gram stain test.
- Gram-positive bacteria** are bacteria that have a single membrane (monoderm) surrounded by a thick peptidoglycan layer. They give a positive result in the Gram stain test.
- Hemostasis** is the process of halting bleeding.
- High-grade dysplasia** is a term to describe precancerous changes that include both individual cell abnormal morphology and abnormal spatial distribution of cells that have abnormal morphology.
- Hyperacute rejection** is the most rapid adverse immunological reaction to a tissue or organ graft typically within 24 h of transplantation, which results in complete failure of function.
- Iatrogenic injury** is tissue or organ damage that has nothing to do with the primary disease and is caused by medical treatment.
- Immune-mediated rejection** is a process in which a transplanted tissue/organ is damaged or destroyed due to an adverse immune response by the recipient.
- In vivo** is a term to describe experiments conducted using animals or humans, as opposed to experiments conducted outside of the body (ex vivo).
- Inflammatory phase** is the second stage in wound healing, characterized by the recruitment of polymorphonuclear leukocytes (neutrophils, eosinophils, basophils, and mast cells), macrophages, and other cells of the innate immune system.

Innate inflammatory cells are cells within the immune system that function by identifying and eliminating potential pathogens.

Ligand affinity is the strength of binding between a ligand and its receptor.

M₁ macrophages have a proinflammatory phenotype that secretes numerous proinflammatory signaling molecules like TNF, IL-1, or IL-6.

M₂ macrophages are antiinflammatory, regulatory macrophages that promote wound healing and secrete antiinflammatory signaling molecules like IL-10 and IL-12.

Matricryptic peptides are bioactive peptides produced by the degradation of parent macromolecules that are part of the natural extracellular matrix (ECM).

Matrix-bound nanovesicles are a class of bioactive, extracellular vesicles localized within the extracellular matrix.

Mononuclear cells are a blood cell type containing a single nucleus, including lymphocytes (T cells, B cells, natural killer—NK cells) and monocytes (which are the precursors of macrophages).

Mucosa layer is the inner lining of tubular organs and body cavities, e.g., the nose, mouth, lungs, and stomach.

Polymorphonuclear cells are the most abundant type of white blood cell in the human body and include neutrophils, eosinophils, basophils, and mast cells. These cells are characterized by varying nuclear shape and are responsible for a variety of immune functions in the body.

Proliferation phase is a stage in wound healing associated with the growth of new tissue. In this phase, angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction occur.

Remodeling phase is the final phase of the wound healing process. Connective tissue is realigned along tension lines, and cells spatially arrange according to functional requirements.

Satellite cells are small muscle progenitor cells found in mature muscle which are involved in the normal growth of muscle and can respond to muscle injury.

Sonication is the use of sound energy to agitate or break a tissue or proteins.

Stricture/stenosis formation is the abnormal narrowing of a tissue cavity in a blood vessel or other tubular structures such as foramina and canals. Stenosis occurs when collagen deposition and smooth muscle cell accumulation is excessive.

Structural molecules are macromolecules that provide shape and form to a tissue.

Tensile strength is the ability of a material to withstand the force of pulling.

T_h1 cells are a class of T-lymphocytes that stimulate a cellular proinflammatory immune response, participate in the modulation of macrophage activation, and stimulate B cells to produce immunoglobulins like IgM and IgG₁.

T_h2 cells are a class of T-lymphocytes that stimulate a humoral immune response, promote B cell proliferation, and induce interleukin 4 (IL-4) production.

Xenogeneic Cells, tissue, or organs belonging to a different species.

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Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Synthetic biomaterials

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6.1 Learning objectives

After reading this chapter you will be able to:

- Describe the molecular structure of various biomaterials and to relate it to material's properties and potential applications in tissue engineering and regenerative medicine.
- Outline how tailor-made biomaterials can be built and modified synthetically.
- Describe and explain the ECM composition, properties and function of the extracellular matrix (ECM), and to propose how to mimic it using synthetic biomaterials.
- Propose novel functional and smart biomaterials featuring tailor-made properties required for applications in tissue engineering and regenerative medicine.
- Critically discuss and appraise the advantages and disadvantages of using synthetic biomaterials in tissue engineering and regenerative medicine.

Few scientists acquainted with the chemistry of biological systems at the molecular level can avoid being inspired.

D. J. Cram, 1988.

The beauty of chemistry is that I can design my own molecular world.

B. L. Feringa, 2015.

The triumphs of engineering skill rest on a chemical foundation.

H. G. Deming, 1936.

6.2 Introduction

Materials are substances used to make objects. A wooden chair, a metal key, a plastic bottle, a rubber ball, leather shoes, a glass window, or a ceramic mug are just a few examples of objects most people use on a daily basis. Yet they all consist of a different material. The choice of the material most often reflects the desired physical properties an object is intended to exhibit. These physical properties are brought about by the structure and chemical identity of the material—this is the so-called **structure–property**

relationship.¹ Based on this relationship, one can select the type of material that best suits their needs. But what if the desired properties are simply not available in the library of existing materials? The known structure–property relationships may also be extended to design novel materials featuring the desired properties. In this process, new chemical entities and structures are proposed with the intended properties in mind. Sometimes the rapidly developing technology will define the demand for a new material and sometimes a newly available material will inspire new technologies. Advances in technology are often either preceded or followed by advances in medicine. Special materials, intended to function and interact with a living organism, are referred to as **biomaterials** and may be an essential part of a biomedical and, more specifically, **tissue engineering** or **regenerative medicine** application.^{2,3} In an attempt to improve the quality of life, the role of biomaterials may be therapeutic or diagnostic in nature.

Much like materials in general, the properties of biomaterials are in large part determined by their chemical identity and structure. In order to function as a biomaterial, a material must be biocompatible; it should not be rejected by the host organism, or instigate any adverse effects on the host or on the implanted material or device. As such, a biomaterial should also be resistant to corrosion in any form. It is worth noting, however, that some biomaterials are specifically designed to undergo degradation upon implantation, in which case the released degradation products should be biocompatible too (see Chapter 7 Degradation of biomaterials). Some biomaterials need not only exist in concert with the surrounding living tissue but also need to provide a suitable biological, morphological, and mechanical host environment for living cells. This biofunctionality is another key feature of a biomaterial in order to fulfill a function in the host.^{2,3}

6.2.1 Why are biomaterials important?

Biomaterials adopt indispensable roles in various biomedical applications. The field of tissue engineering and regenerative medicine relies on controlling the behavior of endogenous or implanted living cells with the aim of restoring tissue or organ function. The role of biomaterial-based substrates here is to provide support for these processes in the form of an artificial ECM. As such, biomaterials are intended to provide a three-dimensional porous, yet chemically and mechanically supportive, structure necessary to accommodate the cells while maintaining interaction with surrounding tissues. For example, this substrate should allow the transport of biologically relevant molecules to and from the cells to ensure their ability to thrive.^{2,3}

Biomaterials are also widely used in **theranostics**. Controlled delivery vehicles able to store, transport, and deliver a drug or another bioactive molecule to a desired location in the tissue, organ, or body with the aim of increasing the efficiency of the therapy are often designed and prepared from biomaterials. In the upcoming field of nanomedicine, biomaterial-built nanosized vehicle molecules are dispatched for transport of relevant therapeutics. Finally, biomaterials are also used in diagnostic purposes where they are employed in molecular imaging of disease targets and biological processes.²

6.2.2 Synthetic biomaterials and their features

Biomaterials may be of natural or synthetic origin depending on whether they are naturally occurring like alginate, cellulose, collagen, or silk, or whether they are obtained following a chemical synthetic process. Some **synthetic polymers**, like polyethylene (PE), polyethylene glycol (PEG), or poly(dimethyl siloxane), are prepared from nonnaturally occurring starting materials. Other synthetic

biomaterials, like cellulose acetate or cross-linked alginate-based gels (**cross-linking** is a process in which two or more polymer chains are joined together, generally with the intention of improving the mechanical properties of a material), are obtained following a simple synthetic modification of a natural biomaterial (sometimes also called **semisynthetic biomaterials**).^{3,4} Fig. 6.1 illustrates several examples of commonly used polymeric biomaterials. Various types of polymeric biomaterials are discussed in more detail in Section 6.3.2 below.

The abundance of naturally occurring biomaterials is reflected in the wide selection of physico-chemical properties (for example, hardness, viscosity, porosity, chemical inertness) that are made available by selecting these materials.^{3,4} However, attaining specific properties somewhat different to those offered by naturally occurring biomaterials may prove difficult. Moreover, achieving a completely new set of properties will prove, more often than not, impossible by using these materials. The use of synthetic biomaterials, on the other hand, allows for fine-tuning of the chemical structure of these materials, which in turn can afford a whole spectrum of physico-chemical properties. Guided by the structure–property relationship outlined above, one will be able to synthetically introduce just the right type and number of chemical modifications necessary to obtain the desired set of properties. On top of that, one may choose to synthetically introduce an entirely new chemical functionality (not a naturally occurring one) to obtain new properties not exhibited by natural biomaterials. Completely new physico-chemical properties may change the functionality of the material and allow for new

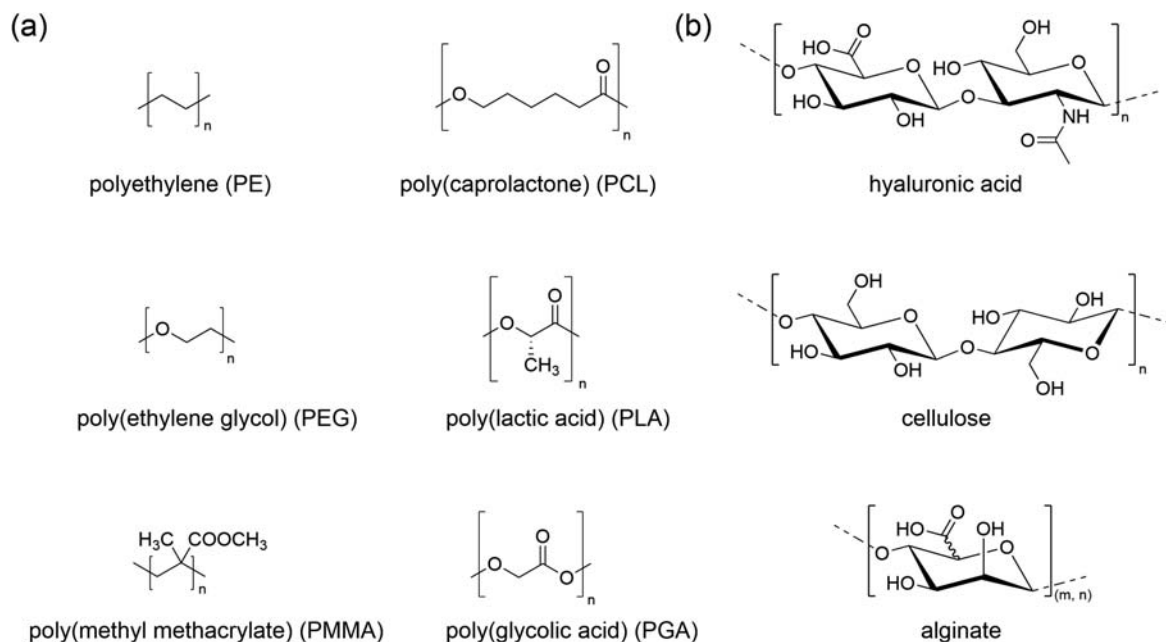


FIGURE 6.1

An overview of examples of biomaterials commonly used in various biomedical applications. (a) Synthetic biomaterials, (b) natural biomaterials.

applications. However, the newly introduced “non-natural” components may also change the material’s interactions with living cells and therefore its **biocompatibility** too (Chapter 8 Cell material interactions deals with this in more detail). In the event that such a tailored material is no longer biocompatible, it can no longer be considered a biomaterial preventing its application in a living organism or in interaction with it. Newly designed and prepared materials always should be checked first for biocompatibility in their intended biological environment.

But which *new* properties may be introduced synthetically? Which properties are desired for applications in tissue engineering? It is actually the properties like **responsiveness** and instructiveness that natural tissues exhibit but natural biomaterials taken out of their natural surroundings do not. Natural tissues (and organs) have the ability to respond to stimuli. For example, your skin will form goose bumps when exposed to the cold, when you are tickled or experience strong emotions. When exposed to water or moisture for a prolonged period of time, you get macerated skin. Both phenomena occur in response to external stimuli and are fully reversible. Other tissues and organs in the human body are also able to demonstrate responsiveness, but this will more often be triggered by a biochemical stimulus that “instructs” that tissue or organ what to do (for example, low or high blood sugar or hormone levels). This behavior is something that is also highly desirable in biomaterials as they are designed to function in concert with the organism that constantly undergoes various biochemical fluctuations. Responsive or instructive materials, the physico-chemical or biological properties of which can be controlled by various stimuli, are therefore very interesting for biomedical applications. Examples of interesting stimuli are pH level, temperature, light, pressure, sugar levels, hormone levels, etc. By means of controlling input (stimulus), one can fine-tune the properties necessary for an application. When a biomaterial is able to autonomously (without deliberate input) respond to its environment, we talk about a **smart biomaterial**.^{5,6} Self-healing biomaterials are a good example of smart biomaterials as they are able to heal or repair their surface in case of damage (scratch or laceration), much like natural skin.

Many naturally occurring and some synthetic biomaterials are biodegradable which means that they degrade upon implantation in the body. Depending on the application and intention, this may be a desired or an undesired feature of a biomaterial and should be taken into consideration when selecting a biomaterial of choice. Chapter 7 Degradation of biomaterials deals with this in more depth.

6.3 Biomaterials and synthetic chemistry: a molecular view

When we think about a material, we usually think about a piece of plastic, metal or ceramic that is hard or even cold to the touch, or a piece of fabric, rubber, or gel that is soft, elastic, or maybe even wet. We notice the material’s color, transparency or opacity, texture, smell, etc. What we often fail to realize, however, is that all of these properties originate from the chemical identity and structure of the material.⁴ But what are actually chemical identity and structure? In order to fully grasp this, we need to zoom into the material to see what it is really made of and how these building elements are connected to each other. Imagine having a microscope, which allows you to zoom into a material as much as you would like. What do you think you would see?

As you start having a closer look at a material, you may begin to notice more physical details on its surface like bumps and dents that you did not notice by the naked eye. These define the surface roughness of the material and are responsible for what you feel when you touch the material. If you take an even closer look, you may see some regular patterns appearing in case the material contains crystalline domains. Fibers may also be observed at this point, for example, in fabrics, as the materials can be made up of these elongated elements. Gels, for example, contain a three-dimensional network spanning through a liquid (water in case of hydrogels) that gives them a gelatin-like soft but still solid texture despite being composed of mostly liquid. By zooming in even more into materials, you begin to recognize individual molecules that make up these networks, fibers, or crystals that the material consists of.⁴

6.3.1 Atoms, molecules, and interactions

Our material world is built up of charged or neutral particles, which sometimes consist of even smaller particles—**atoms**, the smallest “units” of any chemical element. Charged particles are called **ions** and may consist of more than one atom, whereas neutral polyatomic particles are called molecules. Positively charged ions are called **cations** and negatively charged ions are called **anions**. The types of molecules, ions, and atoms making up a material, their relative ratios in a given material, and the way they are all connected to each other, is what defines the material’s chemical identity.⁷

There are three strong interatomic chemical bonds holding atoms or ions together: metallic, ionic, and covalent bonds. All three are based on the electrostatic interaction. Metals, like iron, gold, or titanium for example, contain their respective cations held together by an electron cloud (delocalized electrons) which is called the metallic bond. These materials are electrically conductive due to the freely moving electrons. In their solid form, most pure metals are also crystalline which means that the ions are arranged in highly ordered three-dimensional structures.⁷ Examples of such metallic biomaterials are titanium or gold dental implants.

Most salts, for example, are ionic compounds, which means that oppositely charged ions are held together in a crystal by the ionic bond. In such a rigid structure, the ions are too big to move around (unlike the much smaller electrons in metals) which also results in an electrically nonconductive ceramic-like material. The presence of ions, however, does increase the solubility of these materials in a polar solvent like water (by forming noncovalent interactions with it as you will see below) which means there is ion exchange taking place with the surrounding medium when ionic compounds are used as biomaterials.⁷ An example of this is bone which owes its hardness to the inorganic components of calcium phosphate, $\text{Ca}_3(\text{PO}_4)_2$, and hydroxyapatite, $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Due to a low but not negligible solubility of these minerals in water, calcium ions constantly exchange between bone and the extracellular fluids (the so-called “**chemical equilibrium** conditions” under which no net change in the amounts/concentrations of all components occurs). For these reasons, these biomaterials are also often used as medical implants and devices.⁸

The covalent bond is probably the most important interatomic chemical bond when considering organic molecules like proteins, lipids, or nucleic acids (DNA or RNA) as most atoms in these molecules are held together by this bond. It is most commonly observed between nonmetal atoms like carbon, oxygen, nitrogen, sulfur, phosphorus, and hydrogen and it is characterized by an electron pair shared between two positively charged atomic nuclei. This electron sharing takes place at specific

orientations around the atoms making the covalent bond spatially directional. An important consequence of this is that (covalently bound) molecules have very specific and nonarbitrary geometries in space. For example, this is one of the reasons why our DNA adopts a very specific helical structure. This will in turn also have consequences for the physico-chemical properties of such materials. Quartz is an example of a material consisting of covalently bound silicon and oxygen atoms throughout the structure (each silicon atom is surrounded by four oxygen atoms and vice versa). Diamond is another one consisting of exclusively covalently bound carbon atoms. Since this type of material is characterized by a strong covalent network throughout the whole material, these materials will typically have high melting points as it takes a lot of energy to break up the network.⁷

In the context of materials, it is also possible and even more common to have distinctive molecules in which atoms are covalently bound to each other and these molecules are then held together by non-covalent **intermolecular interactions**, sometimes also called **supramolecular interactions**.⁷ These interactions are typically weaker than the interatomic bonds described above but still have a significant effect on materials' properties. Examples of such naturally occurring biomaterials are collagen protein molecules assembled together to make connective tissue or polysaccharide molecules held together in cellulose. Both materials are made of long chain-like molecules which interact with one another through noncovalent interactions.

An ionic interaction is also possible between two molecules if they carry charged groups (these are then strictly speaking ions) in a similar fashion as described above for the ionic bond. This bond or interaction, however, is not only found in crystals as described above but may also be observed in solution and, as such, it is typically not the only interaction a molecule is involved in. Examples are protein molecules which comprise a series of smaller amino acid molecules covalently connected into a longer chain (polymer). Some amino acids carry charged (ionic) groups and oppositely charged groups belonging to different chains may attract each other and keep the chains together.⁷

When in close proximity, (neutral) atoms and molecules interact too and these interactions are known as the van der Waals forces.⁷ These interactions are caused by the attraction between induced or permanent electric dipoles (temporary or permanent separations of positive and negative charges in an atom or a molecule) and may be more pronounced in one part of a larger molecule (macromolecule) than in another. These forces will typically be stronger between permanent dipoles than between two induced dipoles. An example is that iodine monochloride, ICl, consisting of polar molecules (dipoles), is a solid at room temperature, whereas bromine, Br₂, consisting of nonpolar molecules of approximately the same weight as ICl, is a liquid; this is due to the stronger intermolecular forces between polar molecules which keep them together more tightly. Likewise, the presence of polar groups (like C=O, C=N, C≡N, etc.) in materials will facilitate stronger interactions between chain-like molecules which will also reflect on the physical properties (higher melting points or glassy transition temperatures, higher solubility in polar solvents, etc.). An exceptionally strong dipole–dipole interaction between molecules is achieved when a hydrogen atom (H), covalently bound to a very electronegative atom (meaning it accumulates a lot of electron density on itself) like nitrogen (N), oxygen (O) or fluorine (F), finds itself in close proximity to another such electronegative atom. Such a strong dipole–dipole interaction is called a **hydrogen bond** (···) and may be illustrated as X–H···X where X is typically N, O or F. A strong O–H···O hydrogen bond between water molecules is responsible for some of the remarkable

properties of water like its high boiling point, its high surface tension, and the fact that ice floats on water—these impressive properties enable all of life on Earth as we know it. Our bodies are also made up of about 60% water meaning that water is the basis for the biological and chemical medium where countless biochemical and biological processes like metabolism and cell division take place. All the biologically relevant molecules, like proteins and enzymes, nucleic acids, carbohydrates, and lipids, are in constant interaction with water, which is for the largest part present in the form of the hydrogen bond. As such, water plays a crucial role in determining the spatial structure and therefore also the function of these biomolecules.⁷

All of these noncovalent intermolecular interactions are also possible between groups belonging to the same chain (molecule) and then we speak of intramolecular interactions.⁷ It is important to note that in complex biological systems there will be numerous diverse noncovalent inter- and intramolecular interactions present that all play an important role in shaping these biomolecules and determining their biological functions. Example are protein molecules comprised of a series of amino acid molecules connected into a single polymer chain through the so-called **peptide bonds** (a covalent type of bond). These polar functional groups may form numerous hydrogen bonds with water and with other groups within in the same molecule. Furthermore, the diverse functional groups specific for each amino acid will also engage in interactions with water and other functional groups through ionic interactions, hydrogen bonds and other type of van der Waals interactions. Polar or charged parts of a (macro)molecule which readily interact with water are called **hydrophilic**, whereas nonpolar segments which prefer interacting with other nonpolar groups are called **hydrophobic** (van der Waals interactions between hydrophobic segments are also called hydrophobic interactions). These countless interactions are ultimately responsible for the specific function a protein, or any other macromolecule will exhibit.^{4,7}

Biomaterials will also exhibit noncovalent interactions between polymer chains that will determine to which extent the chains are aligned and packed close to each other which will in turn determine the overall properties of the material like its hardness, viscosity, flexibility, solubility, etc. Fig. 6.2 illustrates

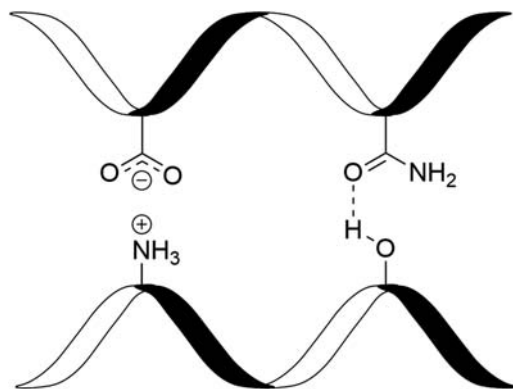


FIGURE 6.2

Examples of noncovalent intermolecular interactions between two polymer chains (ionic interaction on the left and hydrogen bond on the right).

examples of such interactions between polymer chains. One should not forget that biomaterials are materials specifically designed to interact with the human body, which means that interaction of such a material with water is imminent. The presence of hydrogen bonds between a biomaterials and water will therefore influence the three-dimensional structure and orientation of chain-like molecules and in turn also their (bio)functionality.^{4,7} An example of this are the aforementioned hydrogels that are in large part made up of water. It is the water–network interaction that gives the material its shape and structure while still allowing aqueous transport of small molecules and ions throughout the material.^{9,10}

6.3.2 Classes of materials

Polymers: naturals, synthetics, and hybrids

Polymers are high molecular weight macromolecules, which consist of many repeating units (called monomers) bound covalently within a long chain. The extreme size of the polymer chain brings along unique macroscopic behavior compared to small molecules. Polymers can be classified by their source origin (natural, hybrid, and synthetic), physical properties (thermoplastics, thermosets, fibers, and elastomers), structure (linear, branched, and cross-linked or network polymers), and polymerization type (addition and condensation), among others. From the biomaterial point of view, the category commonly used is based on the origin: natural, hybrid (or semisynthetic), and synthetic polymers.

Natural polymers, also called biopolymers, are produced by living organisms. From collagen produced by humans and animals to cellulose synthesized by bacteria and plants, the nature provides us with many biopolymers that make life possible. Two typical examples of biopolymers are proteins/polypeptides (e.g., enzymes, silk, keratin, fibrin) and polysaccharides (e.g., chitin, starch, hyaluronan). Collagen, chitin, and alginate have been widely used in biomedical applications. Even though animal-derived polymers possess cell recognition patterns enabling to stimulate cell response, there is pathogenic risk and immune rejection of their use in human therapy. On the other hand, plant-derived polymers are usually biocompatible, but they lack cell adhesion sites.

Synthetic polymers are prepared by scientists in the laboratory (and scaled up by engineers in factories). The synthetic chemical reaction, which allows monomers (small molecules) to bind covalently, is called **polymerization**. Synthetic polymers exhibit versatile properties depending on the monomer composition. The **rational design** of the polymer allows for the selection of diverse functional groups, and in turn for modulating the mechanical properties, biodegradation rate, etc. Several examples of synthetic polymers commonly used as biomaterials are polyesters (e.g., poly(caprolactone)—PCL, poly(lactic acid)—PLA, poly(glycolic acid)—PGA), polyether's (e.g., poly(ethylene glycol)—PEG), and their combinations (*copolymers*; e.g., poly(butylene-*co*-terephthalate)—PBT). However, the lack of biological cues for promoting cell responsive and their chemical simplicity are major disadvantages of synthetic polymers.

Hybrid (or semisynthetic) polymers are mostly derived from naturally occurring polymers by chemical modifications. The combination of biopolymers with synthetic molecules and/or macromolecules can potentially overcome the limiting properties of both purely natural and synthetic polymers. Ideally, the

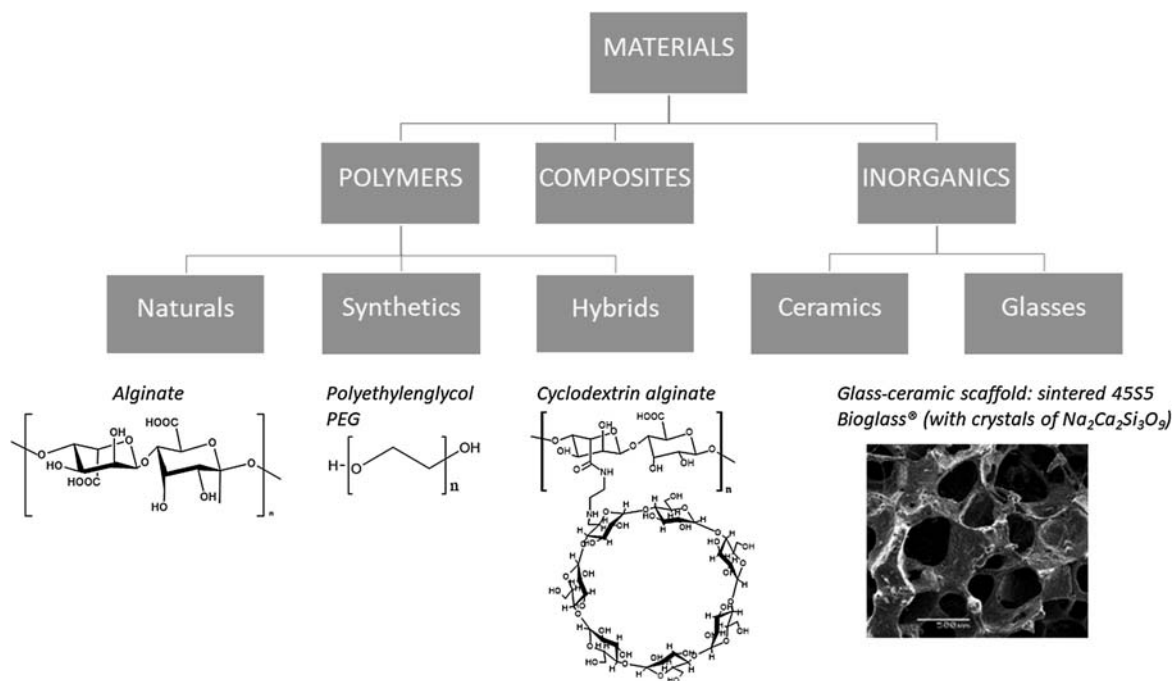
hybrid polymers merge the mechanical and strength properties of the synthetic polymers with the biodegradability and biocompatibility of the natural ones. Synthetic and natural polymers modified with biomolecules are also included in this category. The multiple combinations of synthetic and natural polymers have broadened the range of polymers available for making biomaterials. For example, chitosan is obtained by deacetylation of chitin, which is polysaccharide extracted from the exoskeleton of crustaceans. By modifying only a portion of acetylated amine groups within the polymer chain, we improve the solubility of this biopolymer, and in turn its processability too. Furthermore, the amine groups of chitosan can react with different molecules for further improving their solubility (at pH 7), as, for example, by attaching quaternary ammonium groups (e.g., glycidyltrimethylammonium chloride).¹¹ We can make more complex chitosan by attaching dendritic structures (bulky highly branched macromolecules), which can encapsulate hydrophobic drugs for developing drug delivery systems, without compromising their biocompatibility.^{12,13}

Inorganics: ceramics and glasses

Ceramic materials are inorganic solids composed of metallic and nonmetallic elements. Both the ionic and covalent bonding may be present in these materials. As monomers are the repeating unit for polymers, the unit cell is a repeating unit in ceramics. It is the smallest unit of volume that is repeated/stacked in space to form the ceramic material. The spatial arrangement of atoms depends on the type of bonding, their sizes, and the need to balance the electrostatic charges. Depending on their structure, ceramics can be categorized as crystalline and noncrystalline (amorphous) compounds, and glasses and glass–ceramics (partially crystallized glasses). Crystalline materials display both short- and long-range order in their structure, whereas amorphous ones may only display short-range order. Ceramic biomaterials have been widely used in biomedical applications as orthopedic and dental implants and porous scaffolds for tissue engineering. We can find either biologically inert or active ceramics. Bioinert ceramics, such as zirconia and alumina, have excellent mechanical properties for load-bearing applications, while bioactive ones, such as hydroxyapatite and Bioglass, have potential osteoconductivity. Ceramics and glasses are discussed in more detail in Chapter 7 Degradation of biomaterials.

Composites

Composite materials, commonly called composites, are formed by combining two or more compounds with different chemical and physical properties, without dissolving or blending them into each other. In general, the major component (the matrix) surrounds and binds together fragments or particles of the minor component, which usually is much stronger material (the reinforcement). The matrix is generally based on polymers, while the reinforcement can be either organic or inorganic compounds. The greatest advantage of composites is their strength and stiffness. In addition, composites are found in nature, as, for example, our bones. Indeed, bone is a structural biological composite made of an inorganic part, mainly consisting of hydroxyapatite, and an organic part, mainly consisting of collagen. Therefore, composites are relevant as biomaterials for biomedical applications due to not only their mechanics but also they can mimic native tissues (Fig. 6.3).

**FIGURE 6.3**

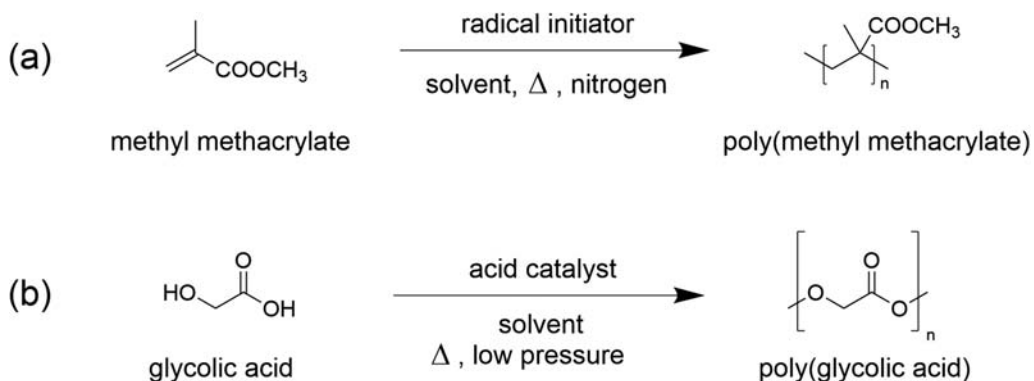
Classification of materials. Examples: alginate, polyethylene glycol, cyclodextrin alginate, and sintered Bioglass. From Chen QZ, Thompson ID, Boccaccini AR. 45S5 Bioglass-derived glass–ceramic scaffolds for bone tissue engineering. *Biomaterials*. 2006;27(11):2414–2425 and Izawa H, Kawakami K, Sumita M, Tateyama Y, Hill JP, Ariga K. β -Cyclodextrin-crosslinked alginate gel for patient-controlled drug delivery systems: regulation of host–guest interactions with mechanical stimuli. *J Mater Chem B*. 2013;1(16):2155–2161.

6.3.3 Synthetic transformations

As mentioned earlier, biomaterials may be of natural or synthetic origin. While natural biomaterials can in principle be harvested from nature, synthetic biomaterials need to be prepared by means of (organic) chemical synthesis.⁷ In this synthetic process, one or more chemical reactions are performed on one or more reactants with the aim of changing their chemical identity as they are converted into one or more products. This usually means changing the composition and/or structure of (a part of) the reactant molecule which will in turn also change its physico-chemical and biological properties.

Synthetic chemistry opens almost endless possibilities to modify existing materials and their properties. This allows chemists to select and carry out transformations necessary to achieve the desired properties. The process of modifying molecules and obtaining target properties may sound easier than it often is in practice. Chemical reactions often need to be optimized to ensure a successful transformation. For example, one may need to find the right reaction conditions (temperature, pH, relative amounts of reagents, reaction time, etc.) to minimize the production of unwanted side-products and maximize the yield of the desired product; this preference of the reaction to give one product and not another is called **selectivity**.^{7,16} Furthermore, the desired product needs to be purified from other participants in the reaction and fully characterized (usually spectroscopically) to unambiguously determine its chemical identity (exact chemical structure). This whole process of optimizing and carrying a chemical reaction, followed by purification and characterization, can be very tedious and time-consuming.

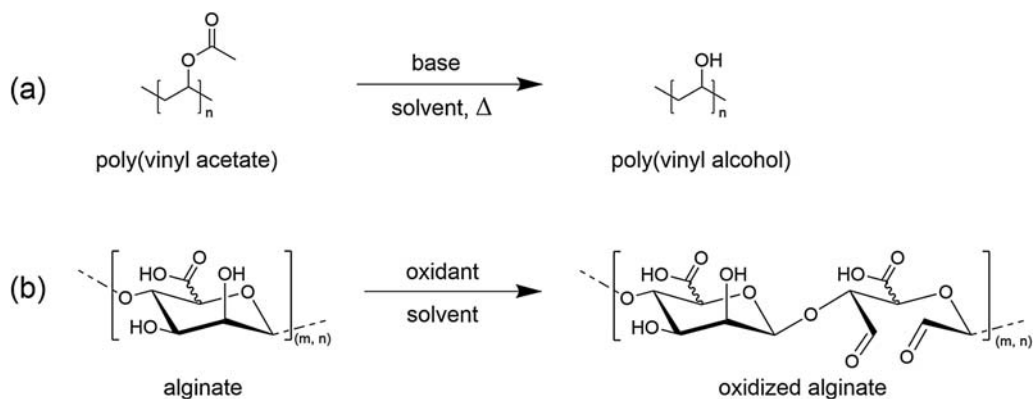
As already mentioned, most organic materials comprise long chain-like molecules (so-called polymers as defined in Section 6.3.2)^{4,16} and many synthetic organic materials can be synthesized directly from small molecules in a process called polymerization in which reactive small molecules, monomers, react with one another to produce a 1D chain or a 2D or 3D network of these monomer units. There are two general mechanisms of polymerization with respect to the manner with which monomers incorporate into a polymer. In a **chain growth polymerization**, there is a growing polymer chain with an active site to which monofunctional monomers are attached one at a time. In the course of the reaction, the average chain length and molecular weight both increase rapidly. The polymerization of **olefins** (organic molecules comprising a C=C bond) typically proceeds through this pathway. Examples of such obtained polymer biomaterials are PE and poly(methyl methacrylate) (PMMA).⁴ In a **step growth polymerization** pairs of bi- or multifunctional reactants of any length (comprising one or more monomer units) combine to form a longer polymer molecule. The average chain length and molecular weight increase rather slowly in this reaction. The formation of polyesters or polyamides from bifunctional monomers typically proceeds through this pathway.⁴ Scheme 6.1 illustrates examples of polymerization reactions used to prepare two commonly used biomaterials, PMMA and PGA from the corresponding monomers.



SCHEME 6.1

The synthetic scheme for the (a) chain growth polymerization of methyl methacrylate to afford poly(methyl methacrylate) (PMMA), (b) step growth polymerization of bifunctional glycolic acid to afford poly(glycolic acid) (PGA).

As opposed to the preparation of synthetic organic materials through polymerization of small molecules, some of these materials can be obtained by synthetically modifying already existing polymer materials (either of natural or synthetic origin). In this approach, functional groups on an existing polymer material undergo a chemical transformation to afford another functional group that will in turn change the properties of the material.^{4,16} Scheme 6.2 illustrates examples of such a modification of a synthetic and a natural biopolymer resulting in biomaterials exhibiting a different range of physico-chemical and biological properties rendering them interesting for a different set of biomedical applications compared to the original biomaterials. The poly(vinyl alcohol) from Scheme 6.2a^{17,18} contains a highly polar hydroxyl group (-OH), capable of forming hydrogen bonds, making this material water-soluble unlike the starting poly(vinyl acetate). The naturally occurring alginate from Scheme 6.2b¹⁹ can be oxidized to

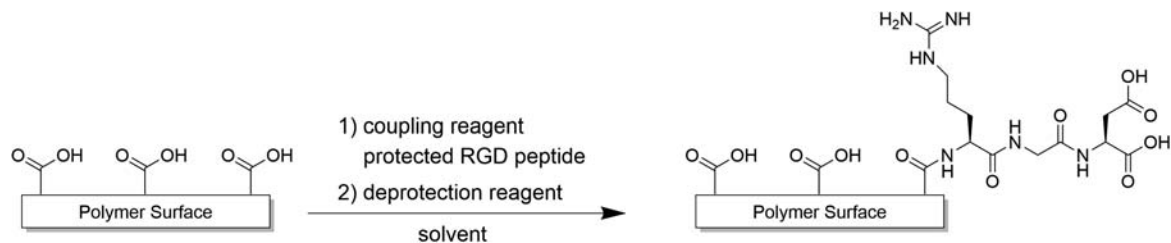
**SCHEME 6.2**

The synthetic scheme for the (a) modification of the synthetic poly(vinyl acetate) to poly(vinyl alcohol), (b) modification of the naturally occurring alginate to oxidized alginate. The biomaterials obtained postmodification exhibit different physico-chemical and biological properties compared to their respective precursor biomaterials and can therefore be used in a different set of biomedical applications.

increase its chemical reactivity (due to the presence of the reactive $-\text{CHO}$ (aldehyde) functional groups). This will in turn enable the formation of dynamic covalent cross-links between individual polymer chains (upon addition of a suitable cross-linker) leading to the formation of gels with special properties (for example, self-healing properties) making it a smart biomaterial.

In order to prepare a novel material,⁴ it may also be necessary to first prepare a polymer through polymerization of a monomer, as illustrated in Scheme 6.1, and then modify the obtained polymer product through an additional chemical reaction as exemplified in Scheme 6.2.

For the purpose of improving biocompatibility and/or cell–material interactions, a biomaterial’s surface may be synthetically modified without altering the material’s interior physical properties. For example, RGD peptides are often attached to biomaterial surfaces to enhance cell adhesion and therefore also the biomaterial’s biofunctionality.^{20,21} Scheme 6.3 illustrates an example of such a modification on a polymer containing carboxylic acid functional groups ($-\text{COOH}$) on the surface.

**SCHEME 6.3**

The synthetic scheme for the surface modification of a polymer with an RGD peptide. The carboxylic acid functional groups ($-\text{COOH}$) on the surface of a polymer (for example, alginate, hyaluronic acid, or poly(caprolactone)) undergo a coupling reaction with the RGD peptide to improve cell–material interactions.

6.4 The extracellular matrix: a chemical view

6.4.1 What are we trying to mimic?

When creating and designing new materials, especially biomaterials, one can look to highly evolved natural systems for inspiration. Nature utilizes complex systems, precision macromolecules, and numerous temporary interactions in order to create the responsive, smart, and functional biomaterials that enable life. Biomaterials can range from the structural actin filaments within a cell, to the collagen fibers outside a cell, to lipid vesicles transmitting signals between cells, to surface coatings on the leaf of a plant. Biomaterials can be found anywhere where there is life.

In this section, we will take a deeper look at the materials outside of a cell, in order to understand how we might rebuild such structures with synthetic materials. While we could choose numerous classes of biomaterials to look at here, we choose to dive into the ECM since it is currently of high importance to tissue engineering. Naturally, cells modulate and communicate with and through the ECM, leading to changes in cellular behavior, differentiation, and tissue maturation. Currently, our field believes that in order to truly control cellular behavior and tissue formation, we must also control and modulate the cellular environment.

In Chapter 5 Extracellular matrix as a bioscaffold for tissue engineering, we learned about the natural components of the ECM, and the strategies within tissue engineering to utilize the natural ECM. As a quick refresher, the ECM is largely comprised of macromolecular proteins, polysaccharides, and proteoglycans, which provide the structural support and environment around cells and within tissue. Comprised of collagen (multiple types), elastin, laminin, fibronectin, and multiple glycosaminoglycans/proteoglycans in combination with minerals, enzymes, and lipid vesicles, from a few key components, the body has evolved to create tissues from the bone to the brain.

The ECM is responsible for five major functions within tissue, and many minor ones which are continually being uncovered. The major functions, as we like to group them, are as follows. (1) *Adhesive substrate*: This is for cells to adhere and interact with, often allowing cells to move through and remodel the matrix. (2) *3D environment*: The ECM provides a 3D niche in which cells can interact and tissue can form. The organization of the ECM often dictates or influences the organization of cells. (3) *Presentation of growth factors*: Several growth factors, biomolecules capable of stimulating cell proliferation, wound healing and occasionally cellular differentiation, are only activated via interactions with the matrix (e.g., TGF- β) and the matrix can control the spatio-temporal organization of such growth factors. (4) *Sequestration of growth factors*: Many of the positively charged growth factors interact with the negatively charged glycosaminoglycans within the matrix, this noncovalent interaction acts as a growth factor reservoir and contributes to this spatio-temporal control. (5) *Mechanical information*: Not only do the mechanical properties (stiffness, **stress relaxation**, strain stiffening) of the matrix influence how cells behave within a tissue, but also the matrix acts as a signal transducer between cells, allowing quick communication via mechanical force.

Then why do we want to recreate something so complex, which already functions as it should? This is a difficult question to answer and comes down to chosen strategy. There are four main reasons why we spend so much time and energy here. (1) *Control*: While cells are good at producing and controlling native ECM, we are not. We are better at controlling and modifying synthetic systems. (2) *Performance*:

hierarchical structures formed from these polymeric macromolecules can be controlled via covalent, supramolecular, and noncovalent interactions. This is the molecular viewpoint of the ECM and brings a complex biological system toward something that a materials scientist and a chemist can start to deconstruct and reconstruct. In this section, we will take a close look at some archetypal examples of ECM components, how they are built, and what lessons we can take away when redesigning synthetic materials.

Collagen is one of the best examples of the combination of polymer design and supramolecular organization found in the ECM. Collagen consists of a linear polypeptide (a poly(amide)) with a tripeptide motif, an extremely simple structure known as an alpha peptide. This collagen polypeptide polymer has an average chain length of 1400 amino acids. These alpha polypeptides assemble into a triple helix (tropocollagen) with molecular weights (M_w) of ~ 300 kDa, driven by hydrogen bonding, facilitated by hydroxyproline residues, and hydrophobic interactions. This tropocollagen in turn is assembled into fibrils of ~ 100 nm diameter, which then further assemble into fibers of 10 micron diameter (in the case of collagen I). This incredibly simple polymer, via postassembly modifications and directed assembly, can ultimately hierarchically create more than 11 molecular networks—from strong and stiff fibrous type I to the soft information rich network type IV.

So, what can we learn from collagen? Well for one, it does not take a complicated polymer in order to have a myriad of functions and impressive properties. Secondly, the supramolecular organization and superstructures formed by macromolecules can heavily influence and dictate their properties and performance. Third, nature smartly uses directed self-assembly (via posttranslational modifications, solubilizing groups, etc.) in order to create complex structures from simpler building blocks. While the first two are often explored in synthetic biomaterials, the last one is more difficult.

Glycosaminoglycans, or GAGs, are also great examples of relatively simple polymers, which can have complex functions in the ECM. GAGs are negatively charged (anionic) polysaccharides. That is, sugar-based polymers with amide groups and negative charges. They are composed of a disaccharide repeat unit and are classified based on this primary linear structure. Heparin/heparin sulfate, chondroitin/chondroitin sulfate, keratin sulfate, and hyaluronic acid are the four main groups of GAGs, each with a different disaccharide motif. These negatively charged linear polymers can provide a wide range of functions, from tissue hydration, to lubrication, to growth factor binding.

So, what can we learn from GAGs? Here, again we see a not-too-complicated polymer, with complex function. With GAGs, we see that nature has optimized these materials for a variety of functions. GAGs are not nearly as organized (supramolecularly and hierarchically) as collagen; thus, this also allows them more flexibility in performing numerous functions.

Bone is another classic example of nature's evolution of materials leading to clues for smarter design. As described above, bone is a classic composite—the marriage of an organic and inorganic materials. Bone consists of highly aligned collagen I fibers offset in layers and intercalated with hydroxyapatite crystals. Collagen, as discussed above, is just a simple polymer, and hydroxyapatite is just a simple inorganic crystal; however, their defined combination and precise organization leads to a natural material with impressive properties. Think of how strong your bones are while being both lightweight and hard. All of this without being too brittle. Our bones have impressive properties, again from simple materials.

So, what can we learn from bone? Yet again we see a simple set of materials that, due to their precise arrangement, have impressive properties. Furthermore, we see that a combination of well-ordered materials with reinforcement from particular or inorganic materials, something that has led to incredible steps forward in engineering materials.

We could go on and on demonstrating the molecular and polymeric view of the ECM components, but by now, we hope you understand. Nature has evolved over millions of years to leverage polymeric materials, precise organization, and supramolecular structures to create the basic materials that build up our body. In deconstructing these materials, what is equally impressive is their simplicity, and the myriad of functions that can be created from this surprisingly basic molecular toolbox.

6.5 Rational design

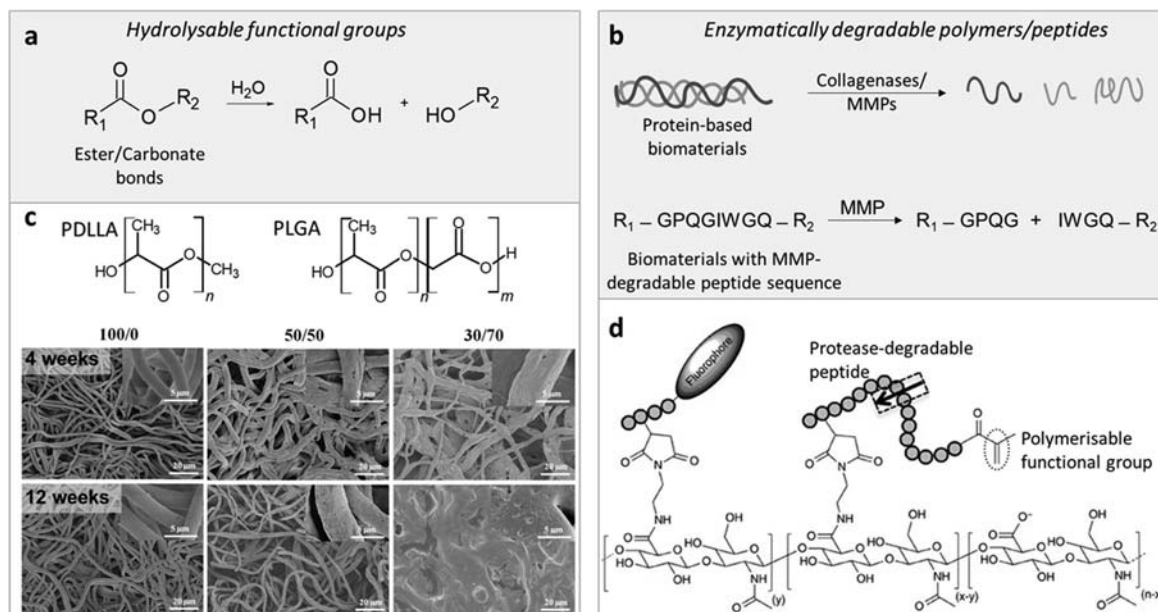
The chemical strategies (which functionalities and where to place them) used for making biomaterials play a key role in their final properties. Therefore, we can design rationally the synthetic biomaterials according to their applications, e.g., hydrogels with RGD peptides to stimuli cell adhesion, biomaterials based on natural polymers for enzymatic degradation. In this section, you can find a summary of the chemical strategies for modulating biomaterial degradation (see [Section 6.5.1](#)), mechanical properties (see [Section 6.5.2](#)), stimuli response (see [Section 6.5.3](#)), bioactivity (see [Section 6.5.4](#)), and biomimicry (see [Section 6.5.5](#)).

6.5.1 Degradation

Synthetic biomaterials should fulfill the biodegradation requirement for medical applications as implants, drug delivery devices, and tissue engineering scaffolds. There are different degradation mechanisms depending on the chemical nature of the biomaterial, morphology, implantation site, among others. The degradation mechanisms and the specific factors that affect biodegradation are detailed in Chapter 7 Degradation of biomaterials. Among the biodegradation mechanisms, **hydrolysis** is one of the predominant for polymer-based biomaterials.

When a synthetic biomaterial will replace a tissue, it is also needed to match the degradation rate in the body to the *timescale of tissue healing*. Therefore, controlling the degradation of biomaterials is a key factor to successful tissue regeneration. The chemical structure of the synthetic biomaterials plays an important role on the degradation rate. Synthetic polymers can be degraded too slowly in physiological conditions, while the natural ones can be degraded too fast.

Most of the natural polymers (e.g., alginate, collagen, hyaluronic acid) degrade through *enzymatic degradation* mechanisms. Protein-based biomaterials are degraded by enzymes collagenases and metalloproteinases, while polysaccharides-based ones by lysosomes and amylases. On the other hand, most of the biodegradable synthetic polymers include urethane, ester, urea, and amide linkages, which degrade via *hydrolytic degradation* mechanism. The types of functional groups, which are present within the polymer structure, affect the degradation rate. Blending, cross-linking and copolymerization have been used to change the degradation rate of both synthetic and natural polymers. For example, the half-life of poly(D,L-lactic acid) (PDLLA)/poly(D,L-lactic-co-glycolic acid) (PLGA) electrospun composites can vary from 4 to 12 weeks just changing the polymer ratio (PDLLA:PLGA) from 0:100 to 50:50 [22](#); see [Fig. 6.5c](#). Blending PLLA with synthetic or natural polymers can also modulate the degradation rate, for example, biomaterials based on PLLA and poly(caprolactone) PCL last for 5 weeks, while blending with collagen degrades 2 weeks.^{[23,24](#)} Only PCL-based biomaterials show a slow degradation (stable for

**FIGURE 6.5**

Degradation of synthetic biomaterials. Hydrolysis (a) and enzymatic degradation (b). Examples of rational design of degradable biomaterials: (c) SEM images of electrospun PDLLA/PLGA composite mats after incubation in PBS (pH 7.4) for 4 and 12 weeks at 37 °C; and (d) hyaluronic acid modified with protease-degradable methacrylated peptide (cleavage site: dotted rectangle) and fluorophore to facilitate monitoring of degradation and in situ imaging. From (c) Zhang E, Zhu C, Yang J, et al. *Electrospun PDLLA/PLGA composite membranes for potential application in guided tissue regeneration*. *Mater Sci & Eng.* 2016;C58:278–285. From (d) Wade RJ, Bassin EJ, Rodell CB, Burdick JA. *Protease-degradable electrospun fibrous hydrogels*. *Nat Commun.* 2015;6:6639.

more than 6 months) compared to PLA and PLGA, making this polymer less attractive for soft tissue engineering but more desirable for long-term implants applications. Blending PCL with chitosan and gelatin increases the degradation rate (3 months), showing potential application for periodontal regeneration.²⁵ Other approach is to add certain protease-degradable peptides within the polymeric structure, which accelerate the degradation rate via enzymatic mechanisms (e.g., mediated by metalloproteinases MMPs). For example, PEG hydrogels with matrix metalloproteinase (MMP)–degradable peptide sequence have been designed for allowing local cell degradation.²⁶ This strategy facilitated degradation of hydrogels and, at the same time, promoted cartilage ECM production. In another approach, the same peptide sequence was added to hyaluronic acid to develop protease-sensitive fibrous scaffolds²⁷; see Fig. 6.5d. The authors have designed a cross-linker containing the protease-cleavable peptide and thiol and methacrylate end groups, which allows reacting with hyaluronic acid and photopolymerization, respectively. They also prepared an electrospun (see Electrospinning in Chapter 11 Scaffold design and fabrication) scaffold combining hyaluronic acid with and without protease-degradable peptide, showing that the degradation was only accelerated when the peptide is present in the polymer structure.

Methacrylation of natural polymers (e.g., gelatin, hyaluronic acid) is one of the most used strategies to modulate the degradation rate, among other properties, by photopolymerization. Gelatin methacrylate (GelMA) has been widely investigated for tissue engineering applications. Its degradation process can

range from hours to weeks depending on the degree of methacrylation and, consequently, cross-linking.^{28–31}

As we described above, there are different chemical strategies for modulating the degradation rate of biomaterials, which are summarized in Fig. 6.5. Therefore, the rational design of the biodegradable biomaterial must take into account the timing for tissue regeneration.

6.5.2 Mechanical properties

Since the beginning of tissue engineering, mimicking the mechanical properties of native tissue has been considered one of the main targets for designing rationally synthetic biomaterials. However, the desired mechanics of tissue-engineering biomaterials has shifted from the static and bulk to the dynamic and surface perspective. Therefore, the stiffness of the construct/scaffold must fulfill the biological mechanics requirements not only in the macroscale (biomechanical integration), but also in the microscale (cell mechano-stimulation). It has been reported that changing the stiffness of the cellular environment led to tremendous differences in cellular responses from simple adhesion to the more complex differentiation. Recently, the development of biomaterials, which mimic the dynamicity of living tissues (*stress relaxation/stiffening responses*), has shown that time-varying mechanics have huge effect on cellular behavior.

Let us first define what *stiffness* is. It is a general structural property, which indicates how much load is necessary to achieve a certain deformation. Stiffness of biomaterials depends on both the material itself and its shape. The main mechanical deformations, which can be tested in biomaterials, are tensile, compressive, shear, and torsion. The mechanical analyses of biomaterials can be performed in a static or dynamic mode, using a mechanical tester or rheometer.³² The moduli obtained from these analyses are related to the biomaterial's stiffness. Typically, static (e.g., tensile, compressive) deformations yield a complex *stress-versus-strain curve*, within which the slope in the linear (elastic) region represents the modulus. On the dynamic analysis, a strain within the linear (**viscoelastic**) region is applied repeatedly over time, in cycles often with changes in frequency or temperature, to yield storage (G') and loss (G'') moduli. The **storage modulus** is related to elastic deformation of the material, whereas the **loss modulus** represents the energy dissipated by internal structural rearrangements. Please check the recommended reading for gaining insight on this topic.

The intermolecular interactions play an important role on the mechanical properties of the synthetic biomaterials. Indeed, stiffness can be controlled by the cross-linking density and type (covalent or physical). Hydrogels based on covalent (nonreversible) polymer network show tunable stiffness by varying the cross-linker concentrations. In the last decades, polyacrylamide (PAAm)-based hydrogels of varying elastic moduli have been explored as cell culture substrates, showing substrate stiffness affects cell adhesion, spreading, migration, differentiation, and proliferation. For example, PAAm hydrogels were proposed for a glomerular basement membrane-mimic platform for studying podocytes behavior affected by chronic kidney diseases.³³ The mechanical properties of PAAm hydrogels correlated with podocyte morphology, elasticity, cytoskeleton reorganization, and podocin expression. This work showed that hydrogel mechanics can be easily modulated (stiffness ranging from 0.6 to 44 kPa) by controlling the cross-linker concentration (Fig. 6.6a and b), and consequently, demonstrating that is an effective strategy to control the cell function.

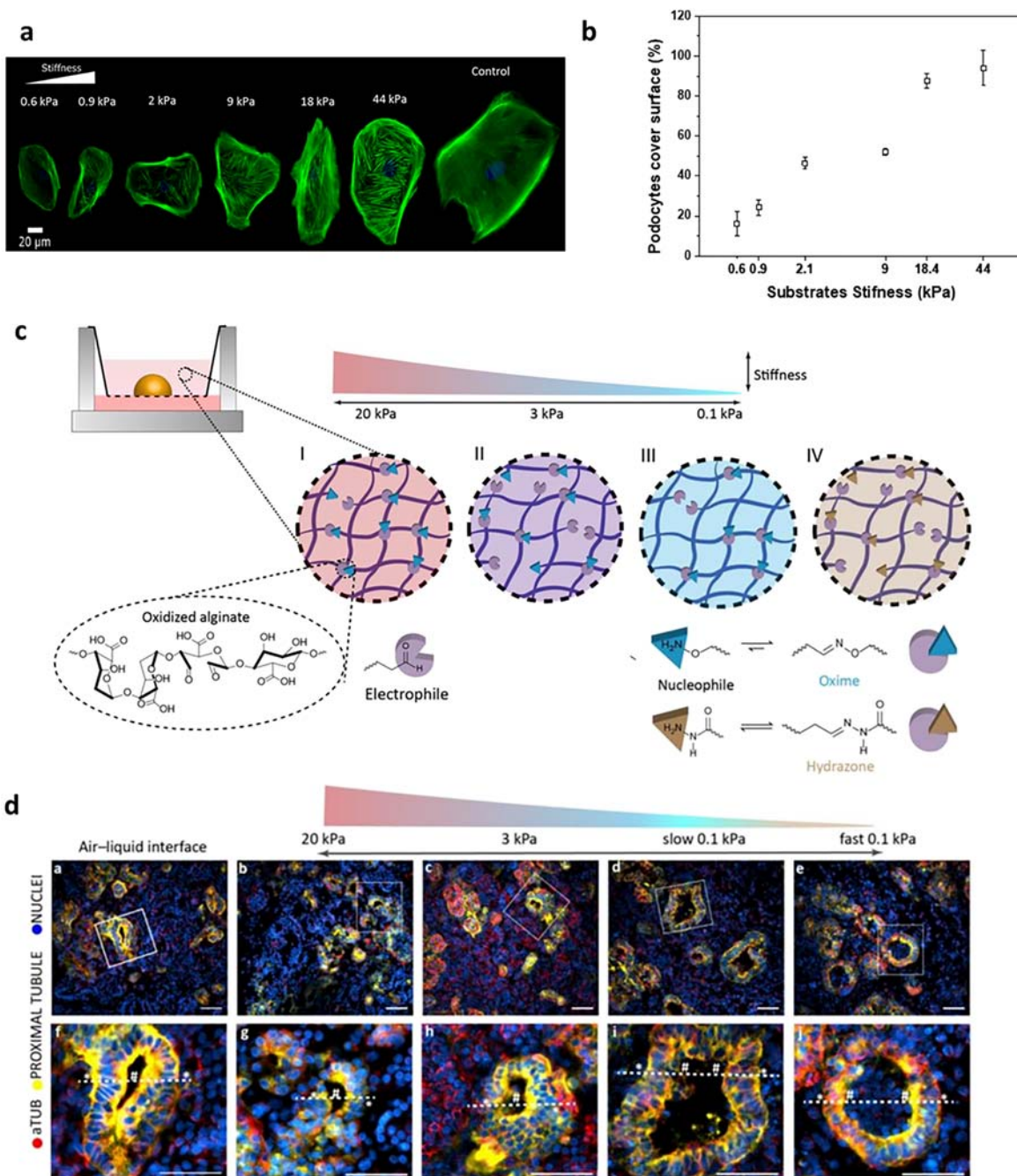


FIGURE 6.6

Designing the mechanical properties of synthetic biomaterials and, hence, the cell behavior. Podocyte cells sense and respond to hydrogel stiffness (a) representative immunofluorescence images of podocytes cultured on PAAm substrates with an elastic modulus ranging from 0.6 to 44 kPa, and (b) percentage of podocytes cover surface as function of substrate's elastic modulus. Stiffness and stress relaxation affect renal phenotype and undesired fibrotic markers for pluripotent stem cell-derived kidney organoids: (c) scheme of the oxidized alginate hydrogels with increased stiffness (similar stress relaxation) by increasing cross-linking degree, or different stress relaxation (similar stiffness) by changing the cross-linker; and (d) images of organoids encapsulated until 7 + 18 d in 0.1 kPa hydrogels (d,i and e,j) showed apical enrichment of LTL⁺ tubules compared to organoids grown in the other hydrogels. The air-liquid interface grown organoids show a clear apical and basal orientated LTL⁺ staining (a,f,k,p). When comparing LTL intensity apical versus basal a significant increase

expression (** = 0.0002 and **** < 0.0001 for slow and fast-relaxing hydrogel respectively; one-way ANOVA) was observed in the soft hydrogels. Scale bars: 50 μm . From (a–b) Abdallah M, Martin M, El Tahchi MR, et al. Influence of hydrolyzed polyacrylamide hydrogel stiffness on podocyte morphology, phenotype, and mechanical properties. *ACS Appl Mater & Inter.* 2019;11(36):32623–32632. From (c–d) Ruiter FAA, Morgan FLC, Roumans N, et al. Soft, dynamic hydrogel confinement improves kidney organoid lumen morphology and reduces epithelial–mesenchymal transition in culture. *Adv. Sci.* 2022, 9, 2200543. <https://doi.org/10.1002/adv.202200543>.

The type of cross-linking chemistry also determines the *space-changing mechanics* of biomaterials. For example, polymeric materials with dynamic covalent (e.g., hydrazone, boronate, thioether) or physical (e.g., hydrogen bonding, guest–host) cross-links show time-varying mechanics (stress relaxation) as found in native tissues. Stress relaxation is a viscoelastic response of a material, which dissipates energy over time after the application of strain.³⁴ Recently, viscoelastic hydrogels have emerged as a step forward to recapitulate the dynamicity of living tissues. Among all reported works, we can find natural (e.g., alginate, hyaluronic acid) and synthetic (e.g., PEG) polymers cross-linked with dynamic covalent bonds, ionic, guest–host interactions, etc. In these materials, viscoelasticity can be modulated independently of the stiffness by controlling mainly the molecular weight of polymer, the binding/association constant of the cross-links, and the cross-linking density. For example, oxidized alginate (alginate with aldehyde groups within the polymer backbone) hydrogels were developed to investigate how hydrogel stiffness and stress relaxation affect renal phenotype and undesired fibrotic markers.³⁵ The authors showed that both stiffness and stress relaxation can modulate cell differentiation (Fig. 6.6c and d). Particularly, the stiff hydrogel led to an absence of certain renal cell types and signs of an epithelial–mesenchymal transition (EMT), whereas encapsulation in soft-stress-relaxing hydrogels led to all major renal segments, fewer fibrosis/EMT associated proteins, apical proximal tubule enrichment, and primary cilia formation, representing a significant improvement over current approaches to culture kidney organoids. Another example, alginate hydrogels, cross-linked with calcium ions (electrostatic interactions), were designed with different stress relaxation, depending on the alginate molecular weight, for chondrocytes culture.³⁶ This work showed that faster relaxation promoted a striking increase in the volume of interconnected cartilage matrix formed by chondrocytes, while slower relaxing gels restricted cell volume expansion and led cartilage degradation and cell death. The authors highlighted stress relaxation as key parameter for cartilage tissue engineering.

6.5.3 Stimuli response

Stimuli-responsive materials undergo a predictable change triggered by specific environmental cues. The biomaterials that are capable to sense signals within the physiological environment are gaining attention for applications in tissue engineering and drug delivery. Any disease or injury always involves *environmental changes*, such as enzymes, pH, temperature, hypoxia, etc. Biomaterials can be designed to sense these intrinsic cues provided by the physiological environments. For example, the wound healing process involves pH changes that can trigger a response in materials, such as antibiotic releasing. The chemical structure of pH-responsive biomaterials includes ionizable groups or acid-cleavable bonds. Banerjee et al. designed poly(*N*-isopropylacrylamide-*co*-acrylic acid) (poly(NIPAm-*co*-AAc)) hydrogel

to deliver vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) in response to a pH change.³⁷ The copolymer composition involves a pH-sensitive monomer (AAc), which triggers a change in the hydrophilicity/hydrophobicity balance of the other monomer (NIPAm) and, consequently, in the hydrogel swelling and growth factors releasing. More information about controlled release strategies is detailed in Chapter 12 Controlled release strategies in tissue engineering.

Biomaterials can also be designed to respond to *external stimuli*, such as light, temperature, ultrasound, or magnetic fields. For example, PEG-based hydrogel was engineered with vitronectin (glycoprotein involved in cell adhesion and spreading) cross-linkers that contained photolabile nitrobenzyl ester (NB) motifs.³⁸ Human mesenchymal stem cells (hMSCs) were encapsulated in these hydrogels and multiphoton lithography enabled precise control over peptide presentation, accomplishing cell differentiation to osteoblasts in a spatially defined manner. Additionally, the authors also reported another step-growth PEG network containing not only NB groups, but also MMP-labile peptide sequence, allowing degradation stimulated by both light and cell-secreted enzyme for fabricating custom endothelialized vasculature.³⁹

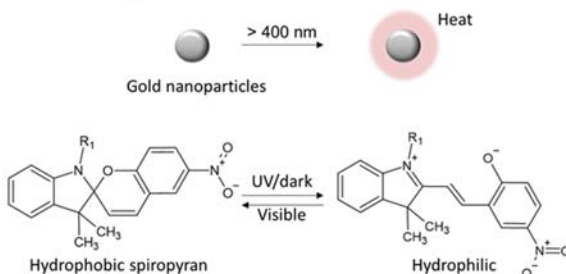
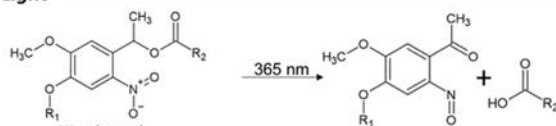
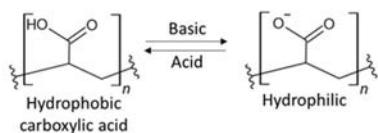
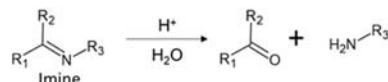
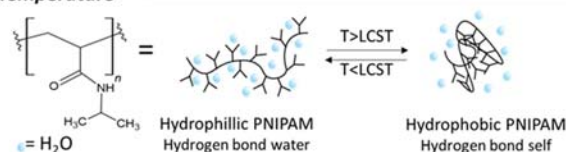
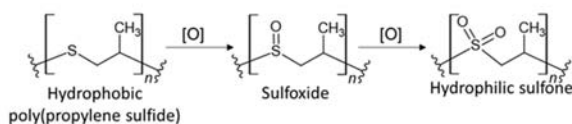
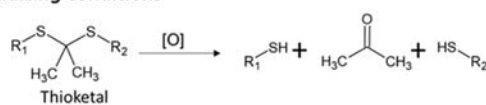
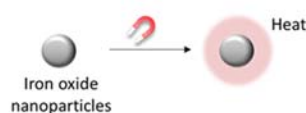
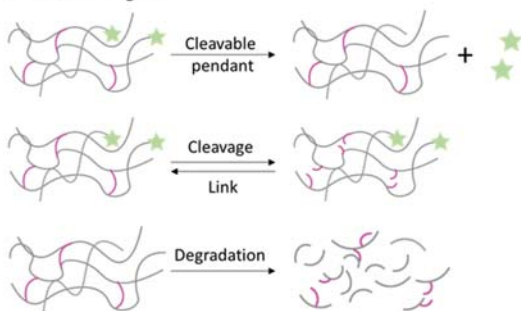
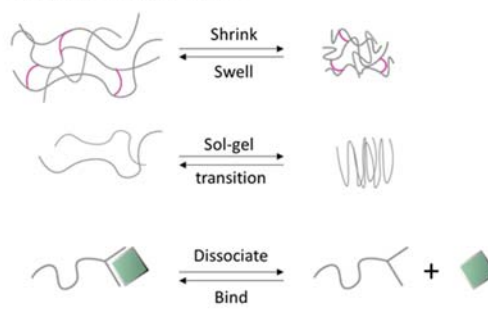
By including certain functional groups, molecule/macromolecules and/or particles within the chemical design of the synthetic biomaterial, we could target specific stimuli responsiveness. Fig. 6.7 shows some typical stimuli-responsive moieties (a) and the typical *mechanisms of response* (b).

Stimuli-responsive biomaterials can be designed using one or more active moieties (Fig. 6.7a) to trigger one or more changes (Fig. 6.7b). In general, pH responsiveness can be achieved by incorporating acid-labile bonds (e.g., hydrazones, imines, acetals, ketals) or ionizable functional groups (e.g., carboxylic acids, amines) within the biomaterial. By the incorporation of water-labile groups (e.g., esters, ureas, carbonates), the biomaterials can undergo hydrolysis (water-induced degradation). For creating thermo-responsive biomaterials, certain polymers (e.g., poly(*N*-isopropylacrylamide) PNIPAM, elastin-like polypeptides ELP, polycaprolactone PCL) undergo a phase transition at certain temperature, which changes the balance of hydrophilicity–hydrophobicity of the biomaterial. High levels of reactive oxygen species (ROS) can trigger a change (e.g., pendant linker or cross-linker cleavage, degradation) in redox-responsive biomaterials, which include oxidative-labile linkers (e.g., disulfide, thioketals, thioethers, arylboronic esters). Ceramic and metallic nanoparticles (e.g., golden, iron oxide) have been explored for developing light-, ultrasound- and magnetic-responsive biomaterials, which can undergo thermal transition, swelling, among others.

6.5.4 Bioactivity

Signaling molecules play a key role on cell-ECM interactions. As it is discussed in Chapter 4 Cell signaling, ECM proteins and diffusible molecules can direct specific a cell's response. While natural ECM components are rich in biochemical information, this is generally absent within synthetic materials. Therefore, synthetic biomaterials have to incorporate signaling molecules in their design to achieve specific cell-responsiveness.

Peptides, proteins, and growth factors can mediate cell adhesion, differentiation, and migration, among other processes. For example, cellular adhesion is typically introduced within synthetic biomaterials

a**Light****pH****Temperature****Oxidizing conditions****Magnetic field****b****Covalent linkages****Non-covalent interactions****FIGURE 6.7**

Toolbox for designing stimuli-responsive synthetic biomaterials. (a) Examples of common stimuli-responsive chemical functionalities employed in synthetic biomaterials. (b) A summary of common mechanisms for stimuli-responsive biomaterials.

through the Arginine-Glycine-Aspartate (RGD) sequence (integrin-binding peptide). The incorporation of VEGFs or bone morphogenic protein (BMP-2) can induce vascularization and bone regeneration, respectively, within biomaterials. The use of cross-links sensitive to MMPs can induce biomaterial degradation during tissue resorption and remodeling. Even the when and where of bioactive molecules is important; the spatial and temporal control of these molecules within the biomaterial affect the cell response. These parameters are discussed in Chapter 8 Cell-material interactions; here we focus on chemical strategies to incorporate the biochemical cues within the biomaterials. The spatio-temporal control of biochemical signals within synthetic materials remains an exciting and challenging area of research.

Proteins and peptides containing lysine or cysteine residues can be easily incorporated within synthetic biomaterials. *Amine groups of lysine* side chains can react with activated ester or carboxylic acid groups of the biomaterial to form amide bonds. The activation of carbonyl groups is typically through 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) coupling. Furthermore, this chemical strategy can be used to introduce a specific molecule/functional group for further reactions within the biomaterial. For example, Fisher et al. functionalized EGF with biotin for immobilization in streptavidin-patterned hyaluronic acid hydrogels.⁴⁰ The authors used a typical NHS-mediated reaction for adding biotin to EGF and then supramolecular interactions between biotin and streptavidin for incorporating EGF within the biomaterial (Fig. 6.8b).

Thiol-bearing cysteine residues can be conjugated to the biomaterial through *thiol-ene click chemistry* or Michael additions, where the double bonds act as an electrophile for reacting with the thiols. For example, an MMP degradable sequence, which includes cysteine at both extreme of the peptide chain, was used as cross-linker for photopolymerization of 4-arm-PEG-norbornene (in this case, a thiol-ene mechanism).⁴¹ This work showed that the hMSCs encapsulated in the MMP degradable hydrogels increased cell-mediated hydrogel degradation, consequently directing cell differentiation.

Careful engineering and rational design of *protein modification* reagents has led to the incorporation of reactive groups in proteins (Fig. 6.8a) for further attachment to materials. These modifications broaden the chemical strategies for further material functionalization with peptides/proteins. It is important to highlight that the environment of the peptides/protein within the biomaterial can also affect its biological activity. For example, an RGD peptide sequence was attached to the two separate networks of a hydrogels.⁴² In this work, hydrogels were made with two networks: one cross-linked by dynamic covalent chemistry (oxidized alginate with adipic acid dihydrazide) and the other one by static chemistry (polymerization of polyethylene glycol diacrylate). Fibroblasts spread quickly on hydrogels with RGD attached to the dynamic network, while cells spread slowly and with spindle-like structure on hydrogels with RGD attached to the static network (Fig. 6.8c and d). Therefore, the rational design of biomaterials with signaling molecules must take into account the chemistry of both the binding and the molecular environment.

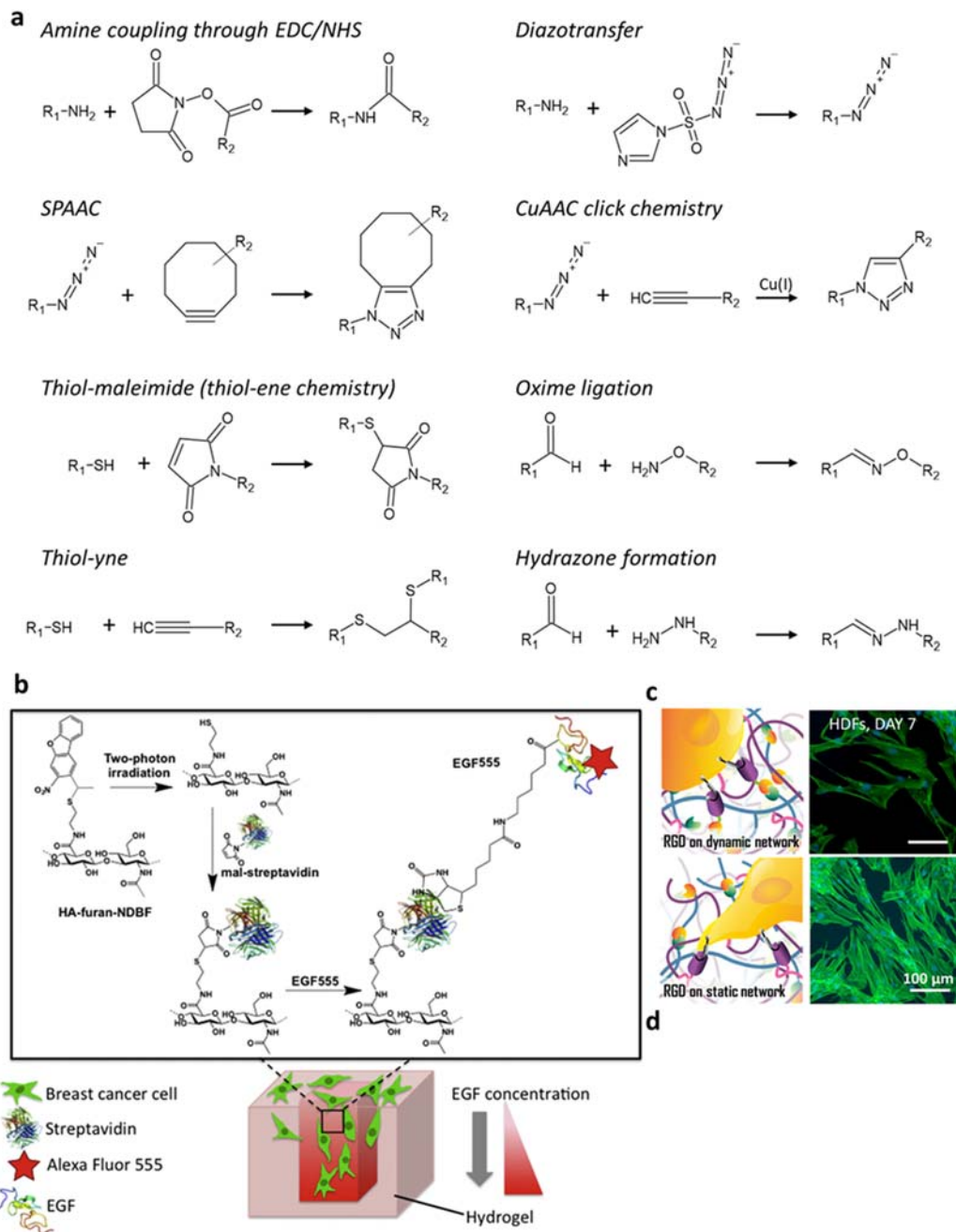


FIGURE 6.8

Incorporating signaling molecules in synthetic biomaterials. (a) Examples of common reactions employed to modify peptides/proteins. Here, R1 and R2 can each refer to a peptide/protein, a biomaterial, or additional functional group. (b) Schematic diagram depicting photopatterning of HANDBF/MMPx hydrogels and subsequent breast cancer cell invasion. Two-photon irradiation of NDBF uncages a reactive thiol, with the concentration of the free thiol proportional to the number of two-photon scans. The free thiol then reacts with maleimide-streptavidin (mal-streptavidin), forming immobilized streptavidin patterns. Biotinylated EGF, modified with Alexa Fluor 555 for visualization (EGF555), binds to the immobilized streptavidin to create EGF gradients. (c, d) The molecular environment of RGD attachment plays a significant role on HDF cells. (c) Images of HDF cells stained with Phalloidin (green, F-actin) and DAPI (blue, nuclei) after 7 days on DN/RGD-dynamic (OA2.5 P10 DN hydrogel with RGD attached to OA network) and DN/RGD-static (OA2.5 P10 DN hydrogel with RGD

attached to PEGDA network). Scale bar: 100 μm . (d) Aspect ratio was analyzed at day 1, 3, and 7 for HDFs on DN/RGD-dynamic and DN/RG-static (N cells ≥ 35 ; N experiments ≥ 2). Statistical significance was determined by the Mann–Whitney test ($*P < .05$, $***P < .001$). Statistical significance given compares same sample at different days and same days different samples. From (b) Fisher SA, Tam RY, Fokina A, Mahmoodi MM, Distefano MD, Shoichet MS. Photo-immobilized EGF chemical gradients differentially impact breast cancer cell invasion and drug response in defined 3D hydrogels. *Biomaterials* 2018;178:751–766. From (c–d) Aldana AA, Morgan FL, Houben S, Pitet LM, Moroni L, Baker MB. Biomimetic double network hydrogels: Combining dynamic and static crosslinks to enable biofabrication and control cell-matrix interactions. *J Poly Sci. November 15, 2021*;59(22):2832–43.

6.5.5 Biomimicry

Synthetic biomaterials have evolved from being just bioinert and mechanically supportive to bioactive materials, which modulate cell behavior. The rational design inspired by the native ECM has led to the *new generation* of biomaterials in tissue engineering. As the previous subsections mentioned, we can design a biomaterial combining different simple chemical strategies for mimicking a target tissue. Indeed, rational design is to take something complex, make it simple, and then rebuild complexity. We are able to create from soft to load-bearing tissue-engineered biomaterials by modulating chemical functionalities, polymer/material composition, cross-linking degree and type, and inorganic particle's loading, among other strategies. Mechanical and degradation properties can mimic native tissues by modulating the chemistry of the synthetic biomaterials, too. Remarkably, the recent works on dynamic hydrogels, using noncovalent and/or dynamic covalent linkages, have shown that dynamic and spatial–temporal complex materials are a promising step toward the recapitulation of ECM functionality and structure.^{43,44,45,46}

The chemical strategies which offer a wide range of opportunities to recreate tissue-engineered biomaterials (as we have shown in this chapter), the type of encapsulated cell lines, and topographical features, among other variables, all matter to approach the complexities of biological systems.

6.6 Future developments

We will not spend too much time on this section, since as soon as this chapter is printed, these words will be out of date. Instead, there are a few main ideas that we would like to bring to attention of the reader, which promise to impact the field further into the future.

6.6.1 Spatiotemporal complexity

Our ability to synthetically design the spatiotemporal complexity found in the native ECM is comically simple. However, this gives us a clear goal to strive toward in the recreation of synthetic systems. Recently, ideas like 3D fabrication, light-based patterning, and responsive materials have given us pathways forward, yet the complexity of the ECM as a molecular system is still something that will challenge scientists for many years to come. During development, tissue homeostasis, and tissue repair, the ECM and the signals surroundings cells and tissue are constantly changing, instructing,

and guiding the proper function. In order to better control the formation and repair of tissue, we need to make significant inroads to match this spatiotemporal complexity in our synthetically designed materials.

6.6.2 Biohybrid approaches

There used to be two camps of biomaterials scientists (okay, many camps, but we simplify). Some believed that synthetic materials were the answer to most problems, while others believed that natural materials were the only promising approach. More and more in the community we start to see a convergence of these two ways of thinking, with an embrace resulting in biohybrid approaches. This can be broadly defined and ranges from synthetic modifications of natural materials (synthetically modified alginate) to biochemically modified or produced synthetic materials (engineered matrices from *E. coli* or enzymatically formed synthetic materials). Either way, the power of rational design and the control afforded by synthesis is becoming more and more combined with the innate biochemical power of evolutionarily optimized natural polymers. Perhaps in the future we can create better materials by combining the two.

6.6.3 Rational design

Rationally designed materials are difficult but are a mainstay of the field. This requires not only deep knowledge generation and reapplication to new situation, but also patience and thoroughness. Ideas like MMP cleavable hydrogels (see above) do not come purely by accident, yet have a major impact on the field. Currently within biomaterials science, there is a large amount of formulation optimization for a desired output. This strategy can help us progress, but is hard to discern rational design from. Yet, this strategy can also be extremely powerful when combined with careful design of experiments and computational/machine learning approaches. Sometimes random walks, with smart analysis, can also give us new design rules. Either way, we urge young scientists going into the field to strive to uncover as much knowledge as possible, to enable a sustainable pace of rational design in the future.

6.6.4 Precision

The balance between precision, disorder, and function in natural biomaterials is truly awe inspiring. A protein, let us say an enzyme, is a poly(peptide) with a precise molecular sequence, activated by post-translational modifications, and folded into an active 3D shape. And what is impressive is that the molecular machinery within a cell enables repeat copies of this enzyme to be nearly identical. We are not yet able to rival this molecular precision in our quest to create well-defined and functional biomaterials. As we gain more control over polymer primary structure, its modifications postpolymerization, and its guided assembly into functional superstructures, we have a promising **design space** which will open up before us. Just remember that the molecular structure of collagen is quite simple, yet via its precision, the posttranslational modifications, and the controlled assembly, it can make numerous networks and forms a basis for most of the materials and tissues within our body.

6.7 Case study: vascularization

The creation and control of vascularization remains a significant challenge within the tissue engineering and regenerative medicine communities. So how can we use or design materials in order to influence angiogenesis? Imagine that you are tasked with the challenge of creating a synthetic material which can allow and facilitate vascularization. What do you do? What do you think of? Where do you turn for help?

6.7.1 How to create a synthetic system for vascularization

So, what is needed for angiogenesis to occur? Well, there are numerous ways to look at this problem; however, there are a few requirements of almost any solution. First, we need a source of cells capable of angiogenesis. These cells can be supplied/implanted or come from endogenous sources. These cells must have something to adhere to, in order to infiltrate and organize into an initial angiogenic network. And, not trivial, this network needs to form in an environment which allows cells to migrate, move, and reorganize. Lastly, these cells need a reason to create new blood vessels, including signals directing them to do so.

In this exercise, we are going to have some limitations. First, the materials must be synthetic or semi-synthetic. Here, we cannot rely on the power of natural materials, but must design something ourselves. Secondly, to make the exercise conceptually simpler, we are going to try to recruit cells from the endogenous environment. This makes regulatory processes simpler but can complicate the readouts that we must do in order to test if our system works.

6.7.2 Designing a material system

One of the first decisions to be made is the reason or the target for vascularization. In many cases, this will limit or narrow the scope, format, and choices to be made. Here, we are going to choose for creating angiogenesis in 3D and look at a general materials approach—not a specific application. Often developing general approaches is more difficult, as the choices to be made can be more open; this freedom can be both a blessing and a curse. The option for a 3D general platform limits our choices to systems like 3D printed scaffolds and hydrogels and pushes us away (though does not exclude) from strategies involving electrospinning or surface coatings. In this instance, we are going to try to design a hydrogel, which is also amenable to 3D printing. This gives us flexibility in the platform.

Okay, now the tougher part. Which material do we choose? There are numerous platform materials capable of forming hydrogels, amenable to 3D printing, and tailorable in their biochemical and mechanical design. The molecular space here is very large: PEG, poly(acrylamides), poly(oxazolines), poly(acrylic acid), poly(vinyl alcohol), and numerous copolymers; do not forget synthetically modified biopolymers like modified alginate, hyaluronic acid, chitosan, and GelMA; we also have options around biomimetic supramolecular systems like small peptides, peptide amphiphiles, urideopyrimidinones, bis-urea's, and benzenetricarboxamides, to name a few. Let us outline a few more choices to be made before we try to make a selection.

We also know that we need the material to be cell adhesive, which means that there either needs to be some intrinsic binding sites for cells (like GelMA) or the systems needs to be able to have peptides ligated to the hydrogel network (like modified alginates or PEG, though the choice of ligation method is often crucial). Furthermore, we know that we need to recruit angiogenic cells from the surrounding tissue with encourage them to infiltrate, and have the right signals to not only organize but start to create vessels. Luckily, a lot of research has shown the power of utilizing VEGF, yet we also see that recent results from the literature suggest a powerful effect from gasotransmitters like H_2S release within materials as well.

A critical parameter we set out was that the network needs to allow for angiogenesis and reorganization of cells throughout the material. This is tougher and means that the hydrogel needs to degrade, have a continuous porous network, or needs to be dynamically reconfigurable, to allow cellular infiltration and organization. Digging into the literature, we see examples where cells can secrete enzymes known as MMPs which can degrade natural materials, and we can see some really clever examples where scientists can engineer in these MMP cleavable sites (via peptides) to allow cell-mediated degradation of synthetic hydrogels. We also see some newer examples where the creation of dynamic hydrogels (with bonds that exchange) can allow for cells to infiltrate without the need for active (or irreversible) degradation of the matrix. Regardless of the strategy, we know that carefully balancing this degradation with new tissue formation is critical for the success from the lab into the clinic.

Now that we have the space sketched out, we can start to make some decisions. The last criteria to decide are on questions like “is this an academic research study, or a clinically translatable solution?” and “are we generating fundamental knowledge, or are we trying to fix a problem?” Different forces are at play with each of these questions, and it is most pertinent that the motivation and goal is clear at the beginning of the design. We will show two instances why, where two very different systems can be designed for these very different goals.

At the end, we hope you can see that there are often multiple paths to be taken, and often it will be environmental or situational factors that help to define the road. While there may be multiple right strategies, making smart decisions at the beginning can ease the journey toward the desired goal.

Clinical/industrial solution

For something to be clinically and industrially relevant, it should ideally be easy, tested, simple, and effective. *Should* is a key word here, as there are always exceptions. Looking back into the literature, we see that the MMP cross-linked PEGs have shown good success for angiogenesis, they are highly mechanically tailorable, can be functionalized with peptides via thiol-ene addition or acrylate polymerization, and have proven success when combined with VEGF. Furthermore, PEG diacrylate is available from several commercial suppliers, is amenable to fabrication, and the peptides and growth factors can also be sourced commercially. Finally, but most importantly, several PEG-based hydrogels are FDA approved, and there is a large body of evidence supporting MMP cleavable

hydrogels, peptide modified hydrogels, and VEGF signaling. This is a good realm for a promising clinical solution.

Yet, there is one problem. There is little room for innovation within PEG hydrogels here; thus, the development of such a system could easily suffer from diminished freedom to operate on any patents generated. Luckily, we will say that we work for/with a company who holds significant intellectual property (IP) in poly(oxazolines), so we choose to port many of the strategies develop with PEG hydrogels over to the poly(oxazoline) platform. And one reminder, as we develop a successful product, the *simplest* working solution is often the best. Industry and medical treatments thrive on simplicity—never underestimate this powerful driving force.

Academic solution

Here, we want to favor generation of knowledge over a rapid path to success. The idea being that by generating more options and better fundamental insight, we can create better solution in the future. For this system, we are going to choose a bioinspired route, and use supramolecular materials in order to try and creation more biomimetic materials for vascularization. Here we choose to use uriedopyrimidinone (Upy)-based hydrogels since they both have some dynamics (benefits cell infiltration and reorganization) and fibrillary morphology (biomimetic for the ECM structure). We will need to figure out how to make these hydrogels cell adhesive (a good academic study), and as a signal, we will choose to use the less explored H_2S to signal the need for angiogenesis. Furthermore, there is not much information on Upy-based hydrogels for 3D printing, so we have a lot of work to do there. Now, one can see that with this design there are a lot of unknowns (cannot go into the literature to find answers), but this is the fun part. There is also a lot of space for innovation and problems to be solved.

Testing

Do not underestimate the sheer amount of testing, optimization, and experimentation that is required for both systems. Neither is quick. While the industrial/clinical solution has a lot of documentation and testing to go into the project to allow for efficient scale-up and reproducibility, the academic solution has a lot of initial failure and searching for conditions before meaningful progress is made. In both systems, the hydrogel needs to be formed, and nearly every variable needs to be optimized to some degree. This ranges from mechanical properties to VEGF/ H_2S loading, to sterilization and manufacturing protocols. Though we chose not to include cells in either strategy as a solution, we still need to test the effect of these properties and optimizations on a relevant cell type. For this, we can use HUVECs, but again here we are forced to often reoptimized based on in vitro data and cellular feedback. As the work continues, we will then need to proceed toward very sophisticated in vitro or in ovo models for angiogenesis (highly preferred—fewer animal studies), allowing us to make very clear and confident decisions on which strategies to take forward to animal studies and human studies with minimal waste of time, money, and life.

Summary

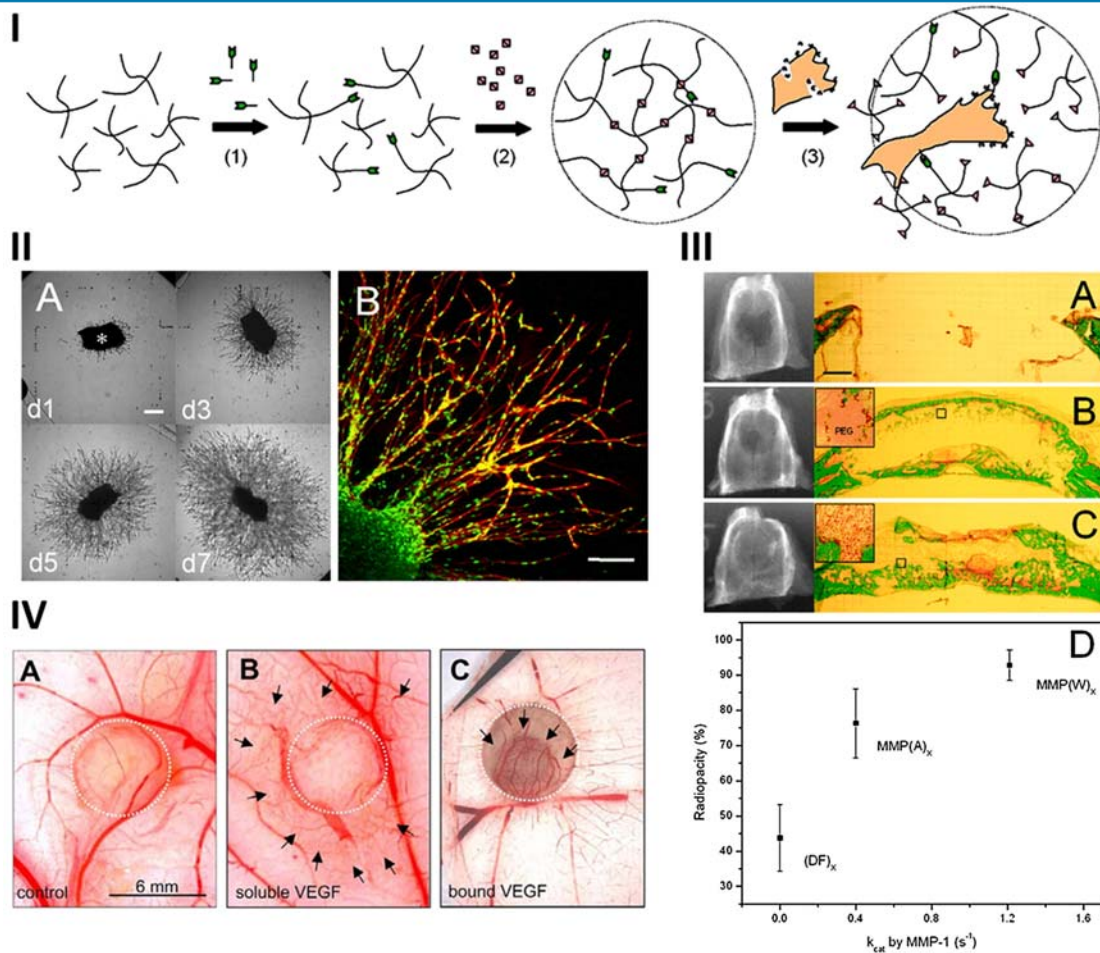
- The molecular structure and chemical identity of the materials determine their properties (structure—property relationship).
- In tissue engineering, a biomaterial is any biocompatible material that interacts with a living organism, which performs, enhances, or replace a function that has been lost through disease or injury.
- Materials are classified by their nature as polymers (naturals, synthetics, or hybrids), inorganics (ceramics and glasses), and composites.
- The physico-chemical properties (for example, the mechanical and degradation properties) of biomaterials can be modulated by chemical synthetic strategies.
- Controlling and modulating the biomaterial's properties and, hence, the cellular environment will allow us to truly control cellular behavior and tissue formation.
- The ECM is a composite material made of polymers and macromolecules assembled by covalent and noncovalent interactions.
- The rational design of synthetic biomaterials is key for bringing closer to desired tissue's properties.
- Synthetic biomaterials which mimic the dynamicity of living tissues have shown a significant effect on cellular behavior.
- Designing smart biomaterials which respond to external or internal stimuli can allow for spatio-temporally control over the mechanical and/or biological cues provided to cells at each stage of tissue regeneration.

Classical experiment

The ability of a material to degrade and allow tissue formation is critical for its success in many tissue engineering and regenerative medicine applications. While classically, hydrolytic degradation of materials has been used, novel ideas around cell-mediated degradation began to emerge in the early 2000s. In one of the classic examples of cell-mediated degradation, researcher chose to utilize one of the cell's own matrix degradation pathways to enable degradation of a synthetic hydrogel. Via the introduction of matrix metalloprotease—sensitive peptides (generally 10–20 amino acids long) as cross-links within PEG hydrogels, invading or encapsulated cells can degrade the synthetic matrix in a biomimetic fashion. A schematic of this hydrogel formation and cell invasion can be seen in

[Fig. 6.9I](#). These MMP-responsive gels showed the ability to allow for fibroblast invasion ([Fig. 6.9II](#)) and facilitated the repair of a mouse cranial defect in an MMP sensitivity—dependent manner ([Fig. 6.9III](#)). In a second study, the authors showed that one could also use these enzyme-sensitive cross-links and linkers to facilitate growth factor delivery from synthetic materials ([Fig. 6.9IV](#)). Here, VEGF was released from an MMP-cleavable PEG hydrogel, and then the VEGF was released via a plasmin-sensitive linker. Two enzymatic events in one material! To date, the engineering of MMP (and other enzymes) cleavable sites within materials is facilitating innovations from clinical solutions to advanced logic gate hydrogels.

Classical experiment—continued

**FIGURE 6.9**

MMP-sensitive synthetic hydrogels enable tissue engineering. (I) Schematic for the formation of the hydrogel and the invasion/cleavage of the MMP-sensitive sites by an invading cell. (II) Fibroblasts invade these hydrogels over time (a, scale bar 250 μ m) and these fibroblasts invaded as groups of spindles (b, scale bar 150 μ m). (III) When implanted in vivo, MMP degradable hydrogels facilitated the repair of a mouse cranial defect. A non-MMP degradable hydrogel (a) and a moderately (b) and highly degradable hydrogel (c) both showed improved defect repair, as can be seen in the quantification of bone density via radiopacity (d). (IV) Incorporation of MMP-sensitive PEG hydrogels with a plasmin degradable linker to VEGF allowed controlled vascularization. Seen is the control (a), the soluble VEGF (b), and the enzymatically released VEGF. (c) Increases in vessel density can be seen around the gel in b, yet is controlled to the location of the gel within c. (VIII) Obtained from Lutolf MP, Lauer-Fields JL, Schmoekel HG, Metters AT, Weber FE, Fields GB, Hubbell JA Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: engineering cell invasion characteristics. *Proc Nat Acad Sci.* April 29, 2003;100(9):5413–8 Copyright 2003 FAESB. (IV) Obtained from Zisch AH, Lutolf MP, Ehrbar M, et al. Cell-demanded release of VEGF from synthetic, biointeractive cell-ingrowth matrices for vascularized tissue growth. *FASEB J.* 2003;17(15):2260–2. Copyright 2003 National Academy of Sciences.

State-of-the-art experiment

While degradation of a material has been established as an important parameter for tissue formation within the material, scientists have also noticed that the native ECM is inherently dynamic. With constant remodeling (breaking down and building back up) of the ECM, it is tough to mimic this dynamic nature in a synthetic biomaterial. Furthermore, we do not really know how a material's dynamics affect cells or tissue formation. Currently, it is still an open question how material dynamics affect cells.

A few key recent studies show that the effect of dynamics might be more important than we thought. Taken together, key studies by the Webber, Stupp, and Dankers labs have paved the way for more fundamental insight into the dynamics needed in synthetic materials in order to facilitate proper tissue regeneration. From facilitating invasion and neotissue formation, to controlling cell adhesion, to matching and enhancing nerve repair, designing dynamics into materials may be a new strategy for the future of regenerative medicine.

Let us take a look deeper into the study by Webber and co-workers. Here researchers used dynamic supramolecular interactions in order to design a cross-linked hydrogel

(Fig. 6.10I-II). Using cucurbiturils and host–guest complexation, they designed hydrogels with different affinity (and kinetics) guests for the host molecules. This led to hydrogels with dynamic cross-links, where they observed a stark difference in tissue formation around an injected hydrogel depending on the dynamics of the cross-links (Fig. 6.10III).

In follow-up studies, the Dankers lab has shown the ability to tune cell adhesion (to RGD) using different dynamics in 1D supramolecular polymers, establishing that cellular adhesion kinetics have a certain threshold. By incorporating monomers with different exchange rates in a supramolecular polymer (Fig. 6.10IV), they found that the slower exchange rates supported cellular adhesion the best (Fig. 6.10V).

Taking this idea further, the Stupp lab recently showed impressive in vivo spinal repair with optimized dynamics within a supramolecular hydrogel. The field is finding out that dynamics of materials matter a lot to tissue engineering and repair, and this promises to be an interesting area of innovation in the near future.

State-of-the-art experiment—continued

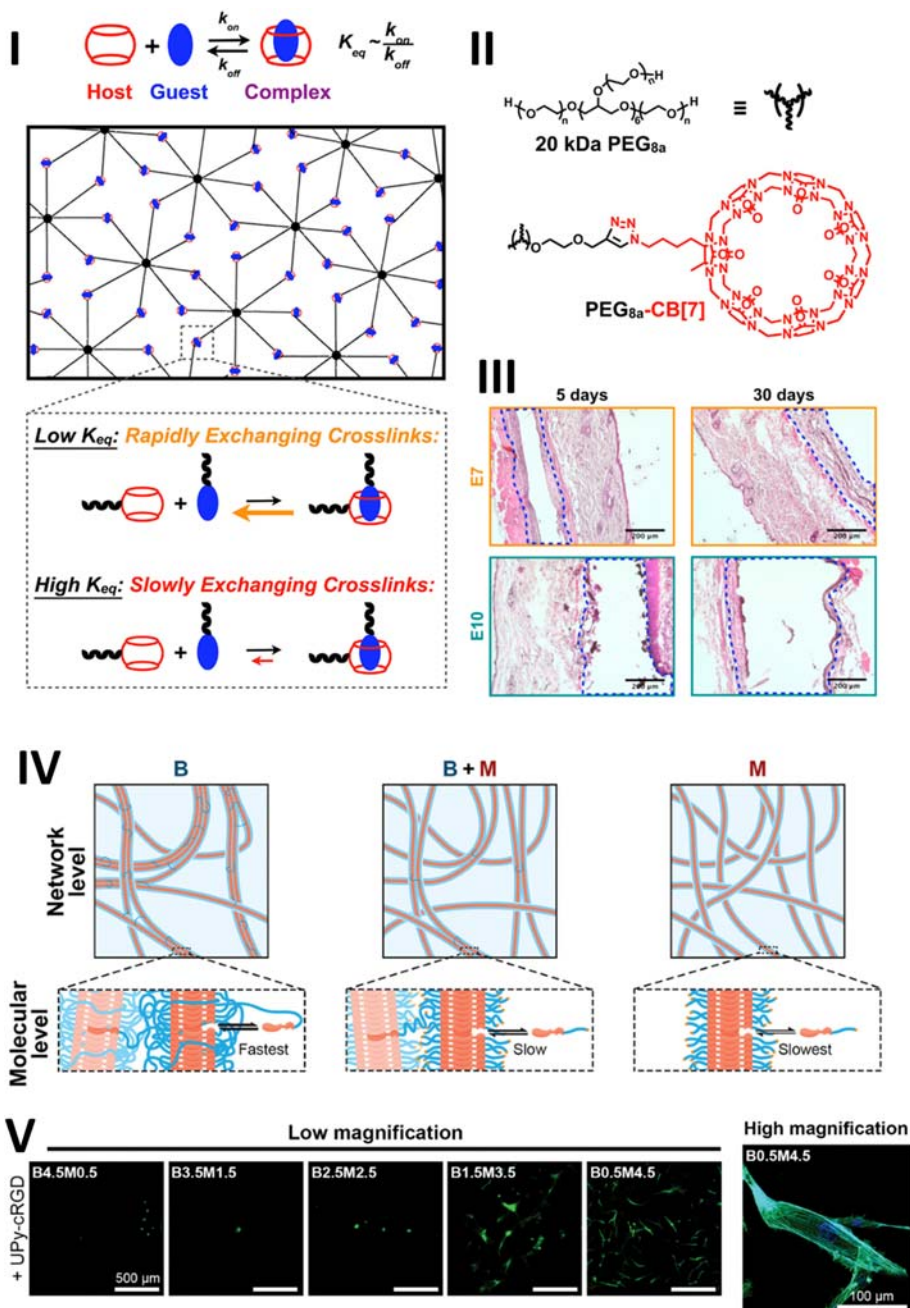


FIGURE 6.10

Dynamics within materials have an impact on their potential for tissue engineering. (I/II) Obtained from Zou L, Braegelman AS, Webber MJ. Dynamic supramolecular hydrogels spanning an unprecedented range of host-guest affinity. *ACS Appl Mater Interfaces*. 2019; 11(16):5695–5700. Copyright 2019 American Chemical Society. (IV and V) Adapted from Diba M, Spaans S, Hendrikse SIS, et al. Engineering the dynamics of cell adhesion cues in supramolecular hydrogels for facile control over cell encapsulation and behavior. *Adv Mater*. 2021;33(37):2008111 under CC BY 4.0. Alvarez Z, Kolberg-Edelbrock AN, Sasselli IR, et al. Bioactive scaffolds with enhanced supramolecular motion promote recovery from spinal Cord Injury. *Science*. 374(6569): 848–856.

6.8 Recommended literature

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6.9 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter.
 1. What is the definition of a biomaterial?
 2. What is a polymer?
 3. What is a copolymer?
 4. What are the differences between natural, synthetic, and hybrid polymers?
 5. What is the relationship between monomers and polymers?
 6. What is a noncovalent interaction and which ones do you know?
 7. What is a covalent bond?
 8. What is a hydrogel?
 9. What is a composite material?
 10. Why are synthetic biomaterials needed and used?
 11. Name the two main growth types of polymerizations.
 12. What are the five major functions of the extracellular matrix?
 13. Is a protein a polymer? Why?
 14. Name two types of degradation mechanisms within synthetic biomaterials.
 15. What is enzymatic degradation? How can we introduce this into synthetic materials?
 16. What is the storage modulus of a material? Why is it important?
 17. Give one example of a stimuli-responsive polymer.
 18. What is the most used molecular modification to introduce cell adhesion?

19. What is biomimicry?
 20. What is a major consideration in designing solution for clinical use?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:
1. If I wanted to create a hydrogel from poly (ethylene glycol) diacrylate designed to degrade only in the presence of cells, how would I do so? Be as specific as possible.
 2. How does degradation rate of a material relate to the tissue formation/repair? What are strategies to increase or decrease the rate of the degradation within a synthetic material?
 3. What are the similarities and differences between natural polymers in the ECM and synthetic polymers made by chemists?
 4. Describe how collagen, a simple protein chain, can have many different materials forms? Give at least two specific examples.
 5. What are the differences between ionic, covalent, and supramolecular bonds/interactions? What are the requirements and benefits of each?
 6. If I wanted to create a biomimetic material based on the architecture of bone, what could I do? What are essential features, and how can I create these in a synthetic system?
 7. When a wound becomes infected, it often becomes more acidic. How can I design a material to sense and react to this local infection?
 8. Recreating the biochemical complexity of the native ECM within synthetic materials remains a major challenge. What are some of the most widely used ways to introduce this biochemical signaling? Which areas still need a lot of research and design?
 9. You are designing a polymer biomaterial but are not sure yet how water-soluble it is going to be so you choose a polymer backbone to which you can easily attach various side groups. Which side groups would you attach to increase and which to decrease the water solubility of the final biomaterial? Also, which applications in the human body would favor a water-soluble and which would favor a water-insoluble biomaterial?
 10. You are designing a cell therapy in which stem cells are supposed to be slowly released in the body over time. How do you design a suitable biomaterial for such a slow-release therapy? What do you need to take into account?

Challenge-based learning

Supramolecular hydrogels for cartilage tissue engineering

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Articular cartilage is a tissue with low regenerative capacity which lacks blood vessels and nerves. Therefore, cartilage tissue does not self-regenerate upon injury or as a

result of age-related degeneration. Uncontrolled degeneration of articular cartilage leads to pain, inflammation, and instability of the knee joint. One strategy to repair early and mid-stage degeneration of articular cartilage is the transplantation of chondrogenic cells into the defect site, in the form of either autologous primary chondrocytes or stem cell-derived chondrocytes. Tissue-engineered strategies use biocompatible cell entrapment systems (e.g., hydrogels) to deliver cells into the cartilage defect. Ideally, these materials should support and control the implanted cells

Continued

Challenge-based learning—continued

throughout the regenerative process. The long-term goal of hydrogels in cartilage regeneration is to express the complex biological signals identified in native cartilage's ECM, and to closely mimic the function of native cartilage.

Motivation and stakeholders

Mechanical signals play an equally important role in tissue regeneration as biochemical signals. Surprisingly, matching these mechanical properties in a synthetic system remains a challenge. Incorporating proper mechanical properties into cell entrapment system like a synthetic hydrogel is of key importance to validate its clinical use in cartilage regeneration. Solutions to mitigate this problem should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as patients with cartilage defects and osteoarthritis, orthopedic surgeons, and the material scientists and bioengineers.

Problem definition

Most hydrogels are composed of a single synthetic polymer chain, which form a simple network, with suboptimal mechanical and biological properties. This is in stark contrast to the ECM of cartilage that contains numerous and precisely organized natural polymers and shows an intricate and highly complex mechanical identity. Multiple polymeric networks interact with each other via covalent and supramolecular interactions within cartilage. To this end, materials to regenerate articulate cartilage should have an adequate polymeric composition to mimic cartilage's microarchitecture and contain bioactive signals to guide the cells in the regeneration process.

Challenge

To incorporate the mechanical properties of the native ECM into synthetic hydrogels using supramolecular chemistry.

Learning scaffold

Reading the Synthetic Biomaterials and Bone/cartilage Tissue Engineering chapters and related literature will help you to understand the following:

1. What defines a hydrogel.
2. The descriptive terms to define the mechanical properties of a material.
3. The mechanical and histological properties of cartilage.
4. Which natural polymers contribute to the mechanical properties of articular cartilage.
5. Cartilage homeostasis and regeneration.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

6. Strategies to manipulate the properties of a hydrogel using covalent chemistry.
7. Strategies to manipulate the mechanical properties of a hydrogel using supramolecular chemistry.
8. The molecular building blocks to engineer tough and resilient hydrogels.
9. Materials and synthesis strategies to engineer tough mechanical properties into complex biomaterials.
10. The advantages and disadvantages of covalent versus supramolecular chemistry in biomaterial synthesis.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

6.10 Glossary

Anions are negatively charged ions with more electrons than protons.

Atoms are the smallest “units” of any chemical element.

Biocompatibility is the ability of a material to perform its function with an appropriate host response without causing (any) undesirable local and systemic effects.

Biomaterials are substances that have been engineered to interact with biological systems for a medical purpose, either a therapeutic (treat, augment, repair, or replace a tissue function of the body) or a diagnostic one.

Cations are positively charged ions with fewer electrons than protons.

Ceramic materials are inorganic solids composed of metallic and nonmetallic elements.

Chain growth polymerization is a polymerization technique where monomer molecules attach one at a time to the active site of a growing polymer chain.

Chemical equilibrium is the state in which both the reactants and products are present in concentrations which have no tendency to change with time.

Composites are materials formed by combining two or more compounds with different chemical and physical properties, without dissolving or blending them into each other.

Cross-linking refers to the process of joining two or more polymer chains together, usually with the aim of changing the physical properties of a material.

Design space in biomaterial engineering is the sum of all possible chemical or physical properties that a given material system can adopt.

Hybrid polymers are semisynthetic polymers derived from naturally occurring ones after chemical modifications.

Hydrogen bond a primarily electrostatic force of attraction between a hydrogen atom which is covalently bound to a more electronegative atom or group, and another electronegative atom bearing a lone pair of electrons—the hydrogen bond acceptor.

Hydrolysis is any chemical reaction in which a molecule of water breaks one or more chemical bonds.

Hydrophilic substances interact readily with water.

Hydrophobic substances avoid contact with water.

Intramolecular interactions is the interaction between molecules, induced by forces including the electromagnetic forces of attraction or repulsion which act between atoms and other types of neighboring particles, e.g., atoms or ions.

Ions are atoms or molecules with a net negative electrical charge. Ions are different from cations, which have a net positive charge.

Loss modulus is a viscoelastic response of a material, which dissipates energy over time after the application of strain.

Natural polymers also called biopolymers, are polymers produced by living organisms.

Olefins are unsaturated hydrocarbons, compounds that contain only hydrogen and carbon and at least one double covalent bond.

Peptide bond is an amide type of covalent chemical bond linking two consecutive alpha-amino acids from C1 of one alpha-amino acid and N2 of another, along a peptide or protein chain.

Polymerization is the chemical reaction which allows monomers to bind covalently.

Polymers are substances or materials consisting of very large molecules, or macromolecules, composed of many repeating subunits.

Rational design is the strategy of creating new molecules with a certain functionality, based upon the ability to predict how the molecule's structure will affect its function.

Regenerative medicine deals with the process of replacing, engineering, or regenerating human or animal cells, tissues, or organs to restore or establish normal function.

Responsiveness is the quality of a biomaterial to respond to stimuli.

Selectivity is the preference of a chemical reaction to generate one product over the another.

Semisynthetic biomaterials are derived from chemical modification of a natural biomaterial to improve function. E.g., cross-linking of collagen to increase stiffness.

Smart biomaterials respond to stimuli and environmental changes and activate their function in response to the stimulus.

Step growth polymerization refers to a type of polymerization mechanism in which bifunctional or multifunctional monomers react to form first dimers, then trimers, longer oligomers (composed of 3–10 monomers), and eventually long chain polymers.

Stimuli-responsive are polymers that are sensitive to triggers from the external environment, including temperature, light, electrical or magnetic fields, and chemicals.

Storage modulus is a property of viscoelastic materials and refers to the ratio of stress to strain under deformation conditions.

Stress relaxation is the observed decrease in stress in response to strain generated to a material.

Structure–property relationship is the relationship between the structure and the way a biomaterial behaves.

Supramolecular interactions are a class of interactions categorized by their noncovalent character. Examples are the van der Waals forces, pi-pi stacking, hydrogen bonding, metal–ligand coordination, and electrostatic interactions.

Synthetic polymers are prepared by scientists performing chemical reactions in the laboratory and scaled up by engineers in factories. Normally, these are from petroleum sources.

Theranostics are nano-size or molecular-level agents serving for both diagnosis and therapy.

Tissue engineering is the design and fabrication of living replacement devices for surgical reconstruction and transplantation.

Viscoelastic is a property of materials to exhibit both viscous and elastic characteristics when undergoing deformation.

© Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Degradation of biomaterials

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7.1 Learning objectives

After reading this chapter, you will be able to:

- Understand the role of biomaterial degradation in performance of tissue scaffolds and other support structures.
- Identify factors that influence degradation, including composition, geometry, process variables, sterilization, and in vivo environment.
- Provide insight into the key mechanisms of breakdown and resorption for different classes of degradable biomaterials.
- Appreciate the timescales of degradation and the appropriate choice of scaffold biomaterial to support tissue regeneration synchronized with resorption.
- Provide an overview of key in vitro and in vivo techniques for monitoring and characterizing degradation of biomaterials.

Polymer science is mature enough to allow one to think about tailor-making macromolecular compounds aimed at interacting purposely with living systems.

M. Vert, 1989

One of the first reasons for modifying the concept of biocompatibility arose with the development of degradable implantable materials and systems, where a stable equilibrium was emphatically not desired, but where the degrading material had to perform a function before or during a process by which it was degraded and eliminated from the body.

D. Williams, 2008

7.2 Introduction

This chapter will cover synthetic biomaterials, which biodegrade in the body over timescales of relevance to tissue healing. Naturally derived biomaterials, i.e., those derived from animals or plants will not be considered, as these are topics included in other chapters (see Chapter 5, Extracellular matrix as a bioscaffold for tissue engineering).

When considering this topic, it is important to firstly define what is mean by biodegradation in the context of biomaterials and furthermore clarify terminology that has been used to describe this process.

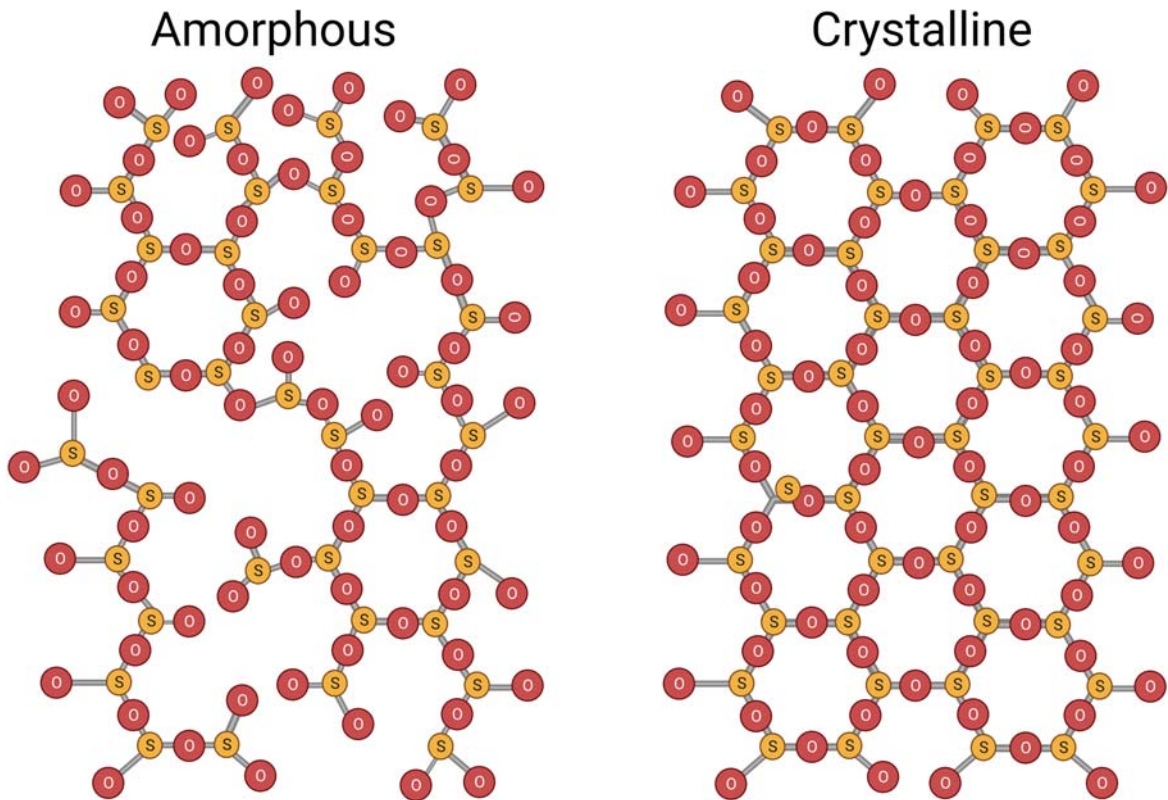
The degradation of a material is its breakdown due to the environmental factors that it is exposed to. For a biomaterial, we consider the environment to be biological and therefore the process termed **biodegradation**. There are a whole host of biological factors that can trigger biodegradation, and these include simple aspects, such as presence of water and oxygen, as well as complex biological processes, for example, relating to inflammatory response, such as phagocytosis. Biodegradation can be either “passive,” where the biomaterial degrades due to the influence of the environment that surrounds it, without interplay with cellular processes in that environment, or “active,” where the degradation products themselves trigger a host cell response, which then actively contributes to degradation. An example of active biodegradation would be where osteoclasts adhere to a synthetic bioceramic and form resorption pits.

The fate of the biomaterial's degradation products is a key consideration in ensuring scaffolds are designed appropriately. This will depend very much on the class of biomaterial, whether it be bioceramic, polymer, or metal. If the degradation products are either integrated within the body or eliminated from the body, then the term to describe this is **bioresorbable**, a specific form of biodegradation. For example, a calcium phosphate bioceramic may undergo dissolution, with the dissolved calcium and phosphate ions becoming integrated into the surrounding bone. A biodegradable polyester may eventually break down into carbon dioxide and water, to be naturally eliminated from the body. Some biomaterials break down into macromolecules or precipitates that remain long-term in the body and are not integrated into normal tissue, for example, the stable oxide product of a biometal corrosion reaction or highly crystalline fragments of residual polymer.

7.3 Bioceramics and glasses

Ceramics are by definition inorganic solid material that contain both metallic elements, e.g., calcium and nonmetallic elements, e.g., oxygen. They are classified according to their structural compounds, A_mX_n , whereby A is the metal, X is the nonmetal, and m and n are the integers. They are generally held together by covalent or ionic chemical bonding. The bonding mechanism accounts for their high compressive strength and hardness and poor ductility and tensile strength. They are generally crystalline or partially crystalline but can also be noncrystalline (amorphous), i.e., an inorganic glass. Crystalline ceramics are characterized by a well-ordered, internal molecular lattice structure (Fig. 7.1). This structure is repeated in a specific tridimensional pattern. The cohesion in crystals is achieved by the binding energy between the atoms in covalently bonded ceramics or by electrostatic forces between anions and cations in ceramics with ionic bonds. Whereas, in amorphous ceramics, the molecules have no order between ions, atoms, or molecules (Fig. 7.1). As a result, the degree of binding energy between entities varies greatly within the solid.

Ceramics that are used in biomaterials applications are called **bioceramics**. They differ from traditional or engineering ceramics in that they have biological functionality and can be safely implanted into the body without invoking a foreign body response. Although, bioceramics are regarded as inherently safe in the body, their physical properties, e.g., particle size, can induce a negative foreign body response. Clinically, bioceramics are mainly used as a bone-regenerative material, particularly in dental and orthopedic applications (see Chapter 16, Cartilage and bone regeneration). The most common bioceramics in bone repair are calcium phosphate (CaP) in the form of hydroxyapatite (HA) and

**FIGURE 7.1**

Amorphous and crystalline structures of SiO_2 (Note: the fourth oxygen for each tetrahedra is not shown). Created with [Biorender.com](https://biorender.com).

tricalcium phosphate (TCP). [Table 7.1](#) summaries some of the bioceramics (and glasses) that are commercially available in the clinic.

Over several decades, CaP, HA, and TCP bioceramics have been found to be biocompatible with good osteoconductive properties, and sometimes osteoinductive, claimed. The mechanisms that support bone growth include: osteoconduction, osteoinduction, and osteogenesis. Both osteoconduction and osteoinduction play a role in osteogenesis, which is the formation of new bone. **Osteoconduction** is basically the growth of new bone on the surface of the old bone matrix (or implant). **Osteoinduction** is the recruitment of immature cells and their stimuli to form preosteoblast cells. When a bioceramic biomaterial is implanted into a bone defect, an additional process occurs known as **osteointegration**, whereby the biomaterial is anchored directly to bone.

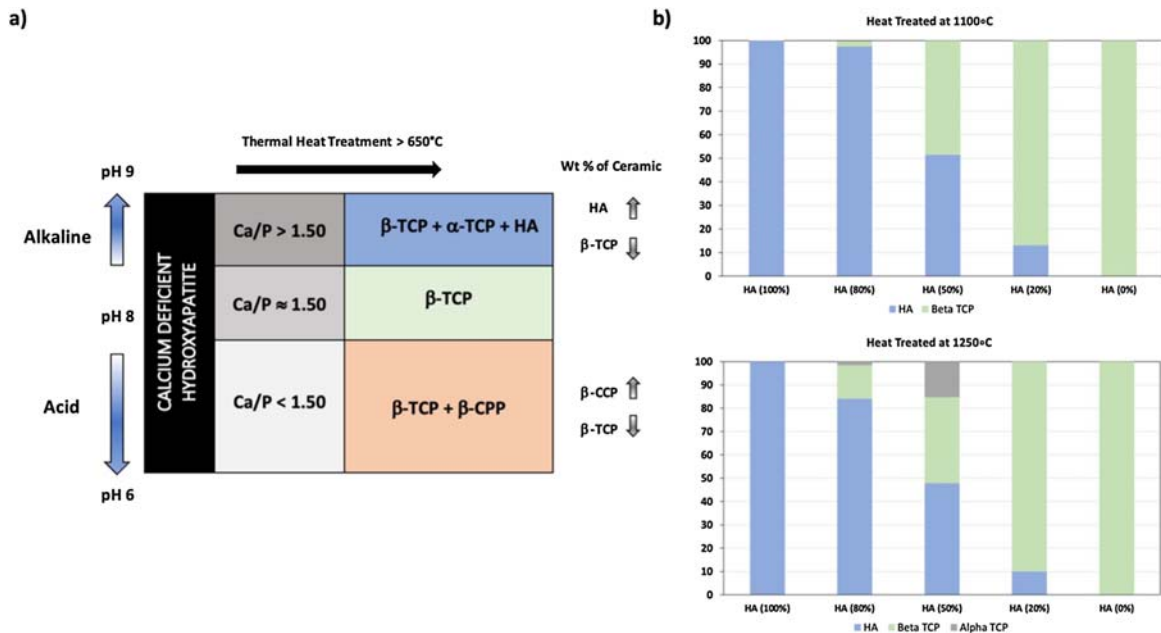
HA is typically a highly crystalline **stoichiometric** apatite, with a chemical formula of $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ and molar ratio 1.67 Ca/P. It contains 39% wt% Ca, 5.18% wt% P, and 3.38% wt % OH. It crystallizes in a hexagonal system (a way to categorize crystalline solids) with a space group of $P6_3/m$ (i.e., sixfold symmetry axis with threefold helix and mirror plane) with specific unit cell

Table 7.1 Examples of some commercially available bioceramics.

Name	Company	Chemistry	Type	Properties	Application
MagnetOs	Kurosbio	Biphasic calcium phosphates (BCPs)	Available in granules and putty	Granules: 65%–75% tricalcium phosphate and 25%–35% hydroxyapatite	Orthopedic
NovaBone	Osteogenics biomedical	Calcium phosphorsilicate (CPS)	Composite (CPS + polyethylene glycol and glycerine binder)	It consists of two particle phases: Phase 1: 90–710 μ bioactive glass particles and phase 2: 32–125 μ calcium phosphosilicate	Dental
Vitoss	Stryker	Beta-tricalcium phosphate (TCP)	Granules	90% porous	Orthopedic
Pro Osteon	Zimmer biomet	Hydroxyapatite and calcium carbonate	Granules, converted coral	$\approx$ 200 μ m interconnected Porosity	Bone graft
PerioGlas (45S5)	Novabone	46.1 mol% SiO ₂ , 24.4 mol% Na ₂ O, 26.9 mol% CaO, and 2.6 mol% P ₂ O ₅	Powders	Bioglass particulate (90–710 μ m).	Dental

dimensions of $a = b = 9.432 \text{ \AA}$, $c = 6.881 \text{ \AA}$.¹ The first documented synthesis of HA was in 1851 by Daubree, who used phosphorus trichloride vapor over red hot lime to produce it. Since then, several synthesis techniques have been developed and optimized, e.g., solid state, aqueous synthesis, hydrothermal. The processing methods used to produce HA, or other bioceramics, e.g., TCP, will influence the physiochemical properties, morphology, chemical composition homogeneity, particle sizes, and degree of crystallinity. All of which affect the ability of the bioceramic to degrade (see Section 7.3.1). Fig. 7.2a shows a phase diagram on how the pH and temperature influence the resultant chemistry in the synthesis of β -TCP with varying Ca/P molar ratios² and its corresponding high-temperature phases (β -TCP, β -CPP, and HA). Fig. 7.2b shows the influence of thermal heat treatment of sample blends of HA: β -TCP heated at 1100 and 1250°C. The graphs show that with an increase in heating temperature from 1100 to 1250°C resulted in an additional phase of alpha TPC in samples with the same initial composition.

Natural HA that is found in bone is nonstoichiometric (typically Ca/P ratio less than 1.67), poorly crystalline, and contains varying degrees of trace elements and ions (e.g., Si, Zn, and carbonate). Over time, HA implant materials have evolved to try to mimic natural HA found in bone in terms of its physiochemical properties. This has led to the development of some interesting and unique graft materials such as marine-derived HA from coral and mineralized algae, coralline.³ Products such as Pro Osteon 500R derived from coral are commercially available; however, their clinical translation has been less successful, despite their unique bone-like properties.

**FIGURE 7.2**

(a) Schematic representation of the influence of the synthesis pH on the Ca/P molar ratio of calcium-deficient hydroxyapatite (CDHA). (b) Shows Rietveld refinement quantitative analysis of X-ray powder diffraction. Adopted from *Chemically pure β-tricalcium phosphate powders: Evidence of two crystal structures*. Le Gars Santi, B., et al., 2021. *Chemically pure β-tricalcium phosphate powders: evidence of two crystal structures*. J. Eur. Ceram. Soc. [10.1016/j.jeurceramsoc.2020.09.055](https://doi.org/10.1016/j.jeurceramsoc.2020.09.055).

Glasses, such as bioactive glass or bioglass, are also commonly categorized as a (bio)ceramic in the literature due to their structure as opposed to their composition. Glasses are amorphous solids with a short-range lattice order, whereas ceramics can be highly crystalline, partly crystalline, or noncrystalline with both short- and long-range lattice order. It is noteworthy that metals can also be made into glasses by extremely rapid quenching processes. So, it should not be assumed that all glasses are ceramics. Glasses are produced by the very rapid cooling of a viscous molten material to a solid state to prevent crystallization. Silica (SiO₂) is the common network forming base for glass, such as the glass-based ceramics developed for bone. **Bioglass** contains SiO₂, CaO, Na₂O, and P₂O₅. The first successful “bioactive” glass material suitable for bone applications was developed by Larry Hench in 1970. He developed bioglass 45S5, which has a composition of 45 wt.% SiO₂, 24.5 wt.% CaO, 24.5 wt.% Na₂O, and 6.0 wt.% P₂O₅ (in weight percentage, wt%). Bioactive glass and its derivatives have been translated into several clinical products by Prof Larry Hench and other researchers and are used for a range of applications including dental applications, ossicular reconstruction, and cancer treatments. Commercial products that use these glasses include PerioGlas (45S5), TheraSphere (Table 7.1). Hench first coined the term “**bioactive**” to describe the interfacial bond between the bioactive glass material and the host tissue that was a result of the biological interaction at the surface of the material, which was later applied to other bioceramic materials. Its ability to bond with bone is directly related to its Ca/P ratio. Bioactive glasses

with a Ca/P ratio substantially lower than 5:1 do not bond to bone. For both bioceramics and glasses, in most applications it is desirable that they degrade over time to allow new bone formation and that the by-product of this process is completely metabolized by the body.

7.3.1 Properties of bioceramics and glasses that influence degradation

Physiochemical and mechanical properties of bioceramics and glasses are important in bone regenerative materials. Here, we focus on their **physiochemical properties** and how this influences their in vitro and in vivo degradation behavior. Ideally, the degradation kinetics of these materials should be synchronized with the ossification of new bone formation. The main physiochemical factors of the material that can influence degradation include:

1. **Crystalline features** (degree of crystallinity, crystal/grain size, grain boundary): The higher the crystallinity, the lower the degradation kinetics. Amorphous biomaterials dissolve faster than crystalline ones.
2. The physical structure and geometry (surface area, surface roughness, porosity, interconnectivity, particle size, granules vs. block): The larger the exposed surface to the solution environment, the faster the biomaterial dissolves, simply because more solid–liquid exchanges can take place.
3. Presence of **additives** (CO_3^{2-} , HPO_4^{2-} , Mg^{2+} , and Na^+ ionic substitution): The presence of some additives of mineral origins within the CaP structure can affect the crystal lattice and therefore can accelerate the dissolution, e.g., carbonate, silicate, or strontium added to HA. The incorporation of carbonate or other ions in CaP may stimulate the carbonic anhydrase activity known to promote the osteoclastic acidic secretion in vitro. On the other hand, the presence of zinc and fluoride in CaP can inhibit osteoclastic resorption in vitro and in vivo.
4. **Chemistry** (chemical composition, chemical bonding): Ionic bonds tend to dissolve better in aqueous solutions, with some exceptions such as calcium carbonate. This is due to its electrostatic bonds between the calcium ion and the carbonate anion, which are too strong for water molecules. Whereas covalently bonding compounds typically do not dissolve in water.
5. **Surface reactivity** (roughness, electrical charge, chemistry, porosity, crystallinity): The reactivity of the surface will influence proteins absorption, cell interaction including signaling and adhesion, chemical interactions, such as the presences of functional groups, e.g., Si–OH in glasses, the surface charge, and the substitution of cationic and anionic within the material.

Crystalline bioceramics generally contain imperfections, whereby (1) additional entities are inserted in the crystal lattice, or (2) some entities can be absent in the crystal, i.e., vacancies. These imperfections result from the materials' processing (e.g., impurities, sintering) and can affect their solubility. In addition, bioceramics are polycrystalline, having less ordered structures at grain boundaries. During the degradation of the bulk bioceramic, these grain boundaries are the first to resorb as they are the "weak-link" and release nano- or microparticles that can trigger biological reactions.

In terms of supporting tissue growth, porous bioceramics are more favorable than dense solids for the following reasons:

- (i) They are more resorbable owing to their large surface area.
- (ii) They support angiogenesis and cellular activity.

- (iii) Macroporosity facilitates bone ingrowth.
- (iv) They provide better anchorage of the graft to the host tissue, preventing fibrous ingrowth and aseptic loosening.
- (v) They induce faster osteointegration, thus improving the mechanical stability of the implant.

Physicochemical dissolution of calcium phosphates resulting from surface reactivity is a result of ionic transfer from the solid phase to the aqueous liquid via surface hydration of Ca^{2+} and phosphate species (PO_4^{3-} , HPO_4^{2-} , H_2PO_4^-) as bioceramic material interacts with its local environment.

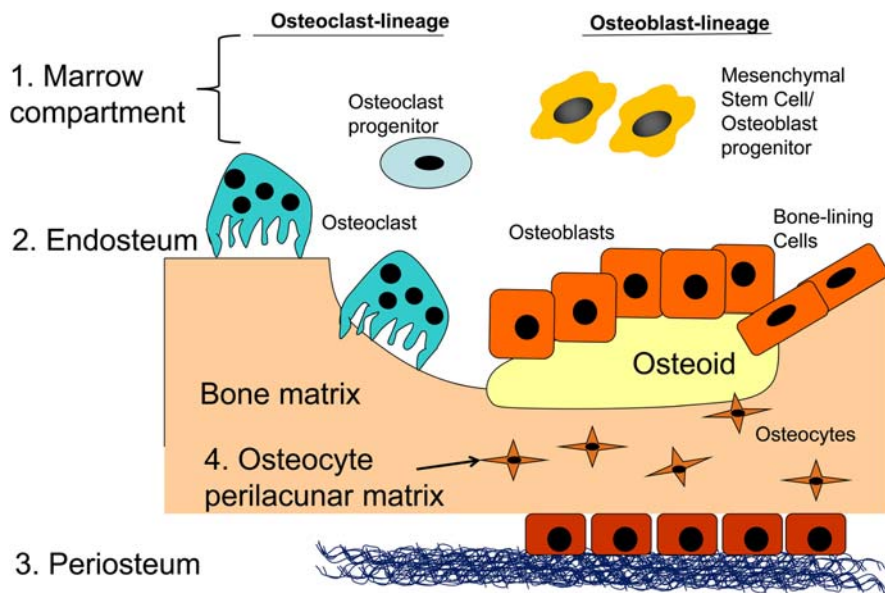
7.3.2 Degradation mechanisms of bioceramics

The degradation kinetics of bioceramics and glasses are an important factor in bone-regenerative medicine for three main reasons. As previously mentioned, to facilitate space for new bone formation, and secondly, it is hypothesized that the biodegradation mechanisms and products have a direct (positive or negative) impact on cells and tissues, thus directing tissue formation. Finally, surface reactivity via degradation/precipitation can influence bonding properties. This is often characterized in terms of the formation of an apatite (phosphate group of minerals) layer on the surface of the bioceramic/glass. The simple reaction sequence that brings about the deposition of apatite later was first proposed by Ref. 4 and later described by Ref. 5. Although, early work by Brown et al. did elude to these type of interactions.⁶ Hench and Ducheyne both describe common steps that are initiated by a chemical reaction at the surface that results in the precipitation of an apatite layer that triggers cell functions and interaction.

In physiologic and artificial aqueous environments, bioceramics can degrade via multiple mechanisms: (1) physicochemical dissolution accompanied by possible phase transformation, (2) cellular degradation mediated by multinucleated cells (MNC), and (3) mechanical fragmentation due to a loss of structural integrity resulting from mechanisms (1) and (2). In biological systems, degradation of bioceramics reflects nonequilibrium processes that occur simultaneously or in competition with each other, involving cellular and the aforementioned physicochemical processes. Cellular processes involve cells such as monocytes/macrophages and osteoclasts that degrade the ceramics by phagocytosis, which is a process by which cells ingest or engulf debris/cells. In bone remodeling, the two principal cells are osteoblasts and osteoclasts. Other cell types are also involved, some of which are shown in Fig. 7.3. Each of these cells produces modulating factors that regulator other cell types. Osteoblasts are bone-forming cells and osteoclasts are bone-resorbing cells. Osteoclast cells can also secrete acid that reduces the pH of that microenvironment causing the old bone matrix or implant to degrade. They also enzymatically cleave bone protein matrix during this process. During bone remodeling, these cells work in synchronicity with one another. Macrophages also play a key role at various stages of the bone repair process, with their most central role being their phagocytic ability.

Physicochemical degradation of bioceramics

The physicochemical degradation of bioceramics can be described as a dissolution–reprecipitation phase transformation process, which is the result of ion exchange at the solid–liquid interface. The kinetics of this degradation process and the surface transformations resulting from ionic exchange depend on the intrinsic properties of the bioceramics (or glass) and the nature of the aqueous environment.

**FIGURE 7.3**

Overview of the cells involved in bone remodeling and the matrix compartments of bone. From *Extracellular matrix networks in bone remodeling*. Alford, A.I., Kozloff, K.M., Hankenson, K.D., 2015. *Extracellular matrix networks in bone remodeling*. *Int. J. Biochem. Cell Biol.* 65, 20–31. ISSN 1357–2725. <https://doi.org/10.1016/j.biocel.2015.05.008>.

From a thermodynamic point of view, CaP ceramics are difficult to dissolve in water but readily dissolve in acids. To study and compare the dissolution kinetics of bioceramics (or glasses) in vitro, physiological saline solutions at different pH levels are used to mimic the alkaline, neutral, and acidic environment found in bone. In these simplified solutions, solubility kinetics correlate with the thermodynamic solubility constant of the bioceramics. There is also an ISO standard (ISO 921 23317) for detecting apatite formation on the surface of a material in simulated body fluids; however, there is differing opinion in the scientific community^{7,8} with respect to the accuracy of this method. More complex supersaturated solutions can be used to more closely mimic the in vivo environment. Supersaturated solutions, such as blood, contain higher concentrations of ions than could be dissolved in water under equilibrium conditions; thus, the supersaturation state is inherently unstable.

The solubility of most of these bioceramics and glasses is well known. An example of the solubility of different CaP phases as a function of Ca^{2+} concentration of the solution and pH under equilibrium conditions at 37°C is shown in Fig. 7.4a. In supersaturation conditions, crystal growth has not yet occurred, but this can be initiated by introducing seeds, i.e., CaP substrates (Fig. 7.4b). Supersaturated solutions frequently used to mimic physiologic solution include cell culture media supplemented with serum or simulated body fluid (SBF).

Although not always the case, it can be generally stated that the physicochemical dissolution behavior of CaP ceramics in vitro and in vivo follows their **thermodynamic solubility**, i.e., $\text{HA} < \text{TCP} < \text{Octacalcium phosphate (OCP)} < \text{dicalcium phosphate dihydrate (DCPD)}$, from least soluble to the most

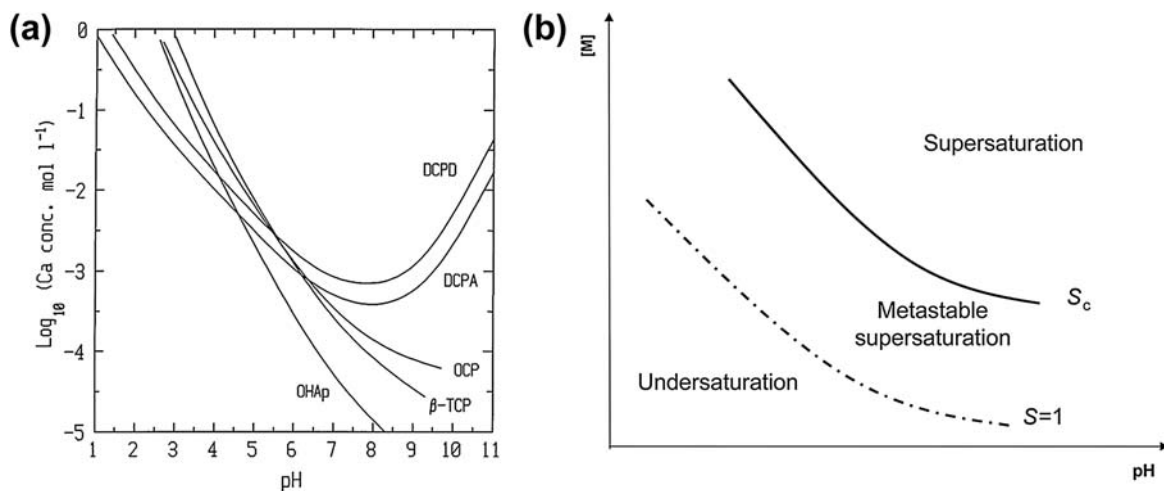


FIGURE 7.4

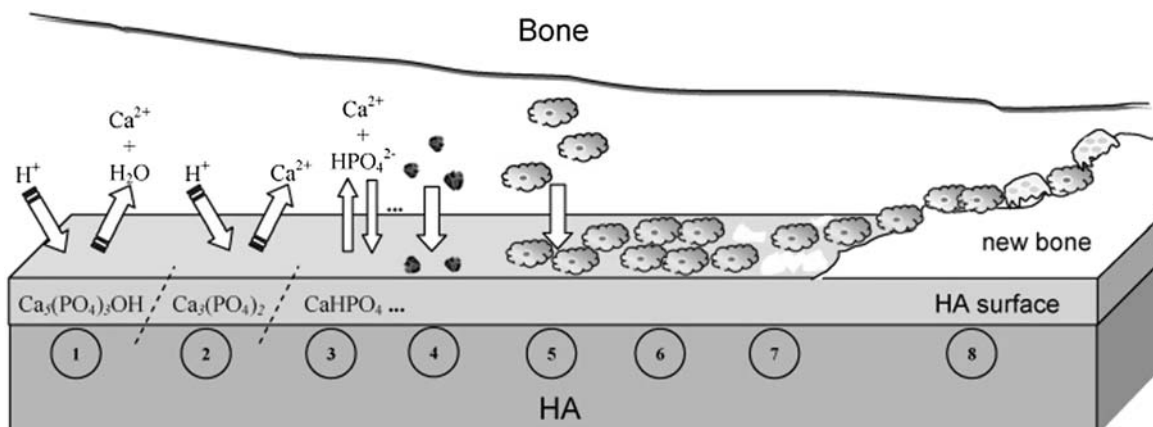
(a) Solubility isotherms of calcium phosphates at 37°C. (b) Solubility isotherms of salts in solution as a function of the pH. This diagram represents the three different saturation states of an ionic solution susceptible to precipitation. From 'Structure and chemistry of the apatites and other calcium orthophosphates', *Studies in Organic Chemistry*. Elliott, J. C., 1994.

soluble. Other additional factors can also affect the physicochemical dissolution kinetics as previously discussed. For example, a wide variety of solubility constants (K_s) have been reported for HA. The solubility product, $K_s = (\text{Ca}^{2+})^5(\text{PO}_4^{3-})^3(\text{OH}^-)$ has been reported to range from 1.8×10^{-58} to $6.3 \pm 2.1 \times 10^{-59}$,⁹ which may be a result of the presence of carbonate, which is known to increase its solubility compared to pure HA.¹⁰

Pure highly crystalline synthetic HA bioceramics are considered to be nondegradable, whereas HA derivatives such as carbonated HA, or β -TCP bioceramics are considered to be degradable. Nonporous HA or other nonporous bioceramics have less solubility than porous structures, as fluid is more restricted and cannot penetrate into the bulk material; therefore, degradation occurs at the outer surface inwards. The ability to produce synthetic porous bioceramics is very challenging, particularly in a nonsymmetrical interconnective porous structure. The bonding mechanism (i.e., ionic, covalent, or both) and energies required to break them will also strongly influence the dissolution of the bioceramics. Crystalline or dense HA is less than ideal for clinical use as both factors can reduce its degradation profile, and its chemical bonds make it overly brittle for load-bearing applications in bone. One way to modulate the degradation profile of CaP bioceramics is by mixing less soluble HA with more soluble β -TCP or incorporating other soluble biocompatible CaP phases. In addition, other CaPs with various solubilities and degradation kinetics are also used clinically to regenerate bone, such as DCPD and amorphous CaP (ACP).

Cellular degradation of bioceramics

A good step-by-step sequence of the events occurs at the ceramic–host interface *in vivo*, shown in Fig. 7.5 by Ref. 11. The events (not listed in time sequence or importance) include: (1) solubilization

**FIGURE 7.5**

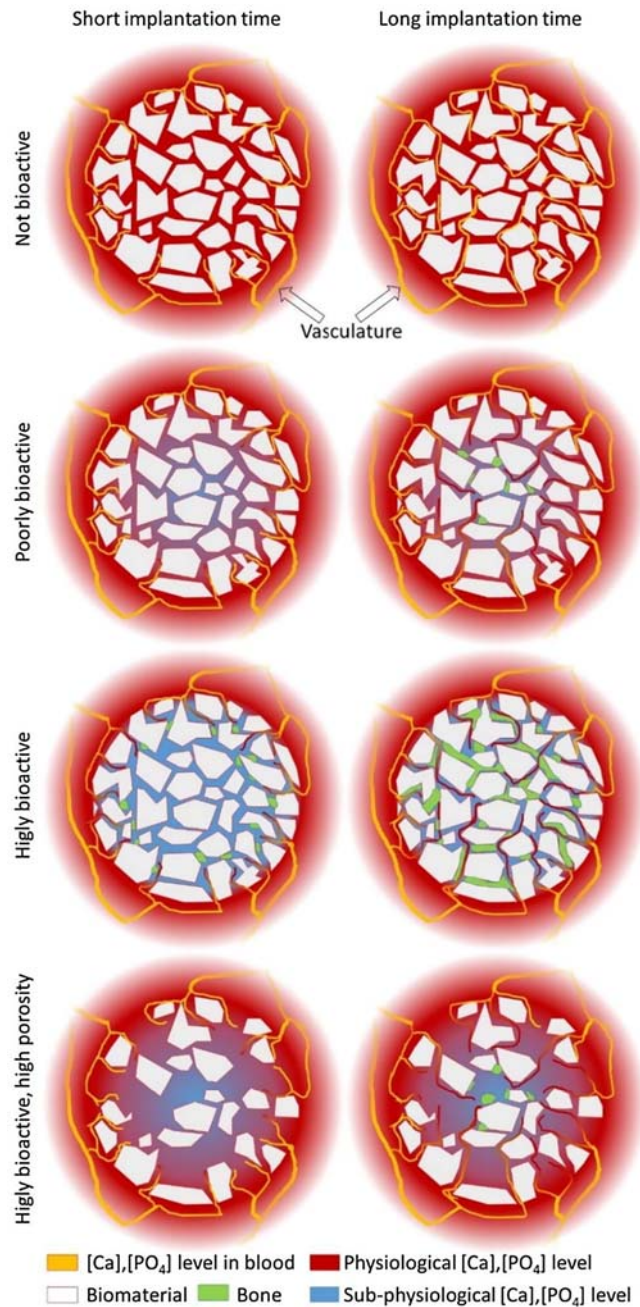
Interactive in vivo of interactions at the surface of the biomaterial. From *Hydroxyapatite surface solubility and effect on cell adhesion*, Bertazzo, S., et al., 2010. *Hydroxyapatite surface solubility and effect on cell adhesion*. *Colloids Surf. B Biointerfaces* 78(2), 177–184.

of the hydroxyapatite surface starts. (2) Continuation of the solubilization of the hydroxyapatite surface. (3) Achievement of equilibrium between physiological solutions and the modified surface of hydroxyapatite. The chemical composition of the surface in this step is indirectly indicated by the equilibrium constant. (4) Adsorption of proteins and organic material. (5 and 6) Cell adhesion and proliferation. (7) Beginning of new bone production. (8) New bone formed and natural bone metabolism.

While surface chemistry is a key attribute in the bioactivity of the bioceramics to induce osteoinduction, bulk material attributes (e.g., porosity, chemistry) are of equal importance, if not critical, e.g., porosity in healthy bone regrowth as highlighted recently by Ref. 12. Fig. 7.6 shows the importance of both bioactivity, calcium and phosphate levels in blood infiltrating and surrounding the biomaterial, and its porosity for long-term healthy bone regeneration. The schematic representation of blood vessel ingrowth and heterotopic bone formation in nonbioactive, poorly bioactive, and highly bioactive granular bone substitutes. The effect of a difference of porosity is also displayed. A higher osteoinduction is expected when the material bioactivity is increased and when the material porosity is reduced (provided blood vessel ingrowth is still possible).

In light of the evidence in recent review, a new definition for bioactive biomaterials has been proposed. “A material is osteoinductive if: (1) it mineralizes in vivo (formation of a “bioactive” apatite layer on the material); (2) it is porous; (3) the pores are large enough to allow blood vessel ingrowth and cell transport into the core of the material; (4) blood supply is insufficient to keep physiological calcium and/or phosphate ion concentrations”.¹²

In terms of the cell interactions, both in vitro and in vivo, it has been observed that osteoclasts can degrade CaP ceramics in a similar way as bone mineral: osteoclasts adhere to the CaP surface and create resorption pits, presumably through acid dissolution of the sealed microenvironment formed between the osteoclast and the material substrate (the “sealing zone”). In vitro, osteoclasts readily adhere and

**FIGURE 7.6**

Schematic showing blood vessel ingrowth and heterotopic bone formation in bone substitutes. *From a proposed mechanism for material-induced heterotopic ossification, Bohner, M., Miron, R.J., 2019. A proposed mechanism for material-induced heterotopic ossification. Mater. Today 22, 132–141.*

resorb several CaP ceramics including HA and TCP. In vivo, multinucleated osteoclast-like cells form microconcavities on the ceramic surface adjacent to newly formed bone and degraded material particulate. In vitro experiments have identified specific physicochemical parameters beyond the primary CaP composition that influence cellular degradation as previously mentioned in the Introduction of this chapter and discussed in more detail below in the context of cellular degradation:

The physicochemical dissolution kinetics: Depending on the synthesis methods, dissolution kinetics can vary widely among CaPs with the same chemical composition. The release of Ca^{2+} influences osteoclastic activity; for instance, above a certain Ca^{2+} concentration, osteoclastic resorption is inhibited. As previously discussed, CaP structure and crystallinity also play a role in dissolution kinetics and therefore may also dictate osteoclast activity.

The presence of additives: The incorporation of carbonate or other ions in CaP may stimulate the carbonic anhydrase activity known to promote the osteoclastic acidic secretion in vitro. On the other hand, the presence of zinc and fluoride in CaP can inhibit osteoclastic resorption in vitro and in vivo. Ions such as strontium have been reported to enhance bone formation, while inhibiting bone resorption.^{13,14}

The surface features: Surface energy was found to modulate the osteoclastic adhesion in vitro. Surface roughness and microporosity appear to enhance osteoclastic attachment and activity.¹⁵

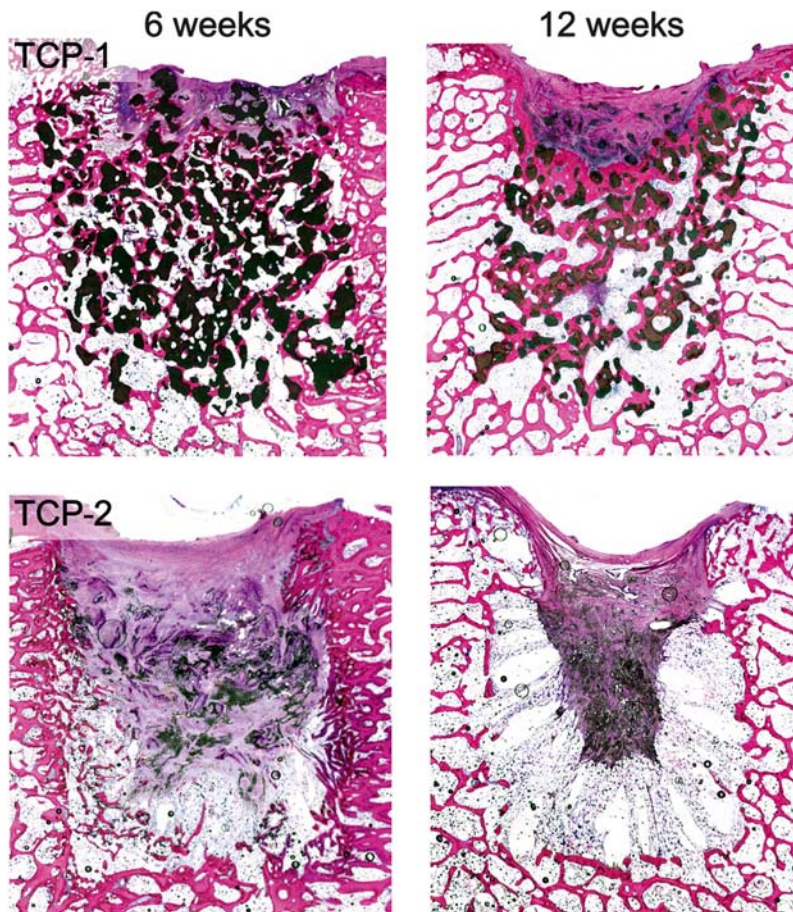
In addition to these parameters, the processing methods (discussed earlier) used to produce the bioceramics can also strongly influence their in vivo performances. To illustrate this, two macroporous β -TCP bioceramics (TCP-1 and TCP-2) of identical composition, which were made by different synthesis methods, were implanted into the femoral condyle of goats. The results shown in Fig. 7.7 demonstrated significantly different in vivo degradation profiles. The figure represents the tunable nature between degradation and bone formation of two β -TCP ceramics with similar composition but made by different synthesis methods. TCP-1 degrades slower than TCP-2 and supports continuous bone ingrowth from the host bone to the center of the implant, reflecting full bone regeneration in the defect. TCP-2 degrades too fast and does not support bone ingrowth; instead, the defect is filled with fibrous tissue. TCP-2 was found to be resorbed too rapidly, resulting in the production of only fibrous tissue growth in the bone defect, while TCP-1 was resorbed much more slowly. This produced significant bone growth in the defect.

7.4 Biodegradable polymers

7.4.1 Introduction

Biodegradable polymers have been used as tissue engineering scaffolds for a wide range of applications within both hard and soft tissues. For successful application, biodegradable polymers must meet several prerequisites. First, the polymer must have mechanical properties and a degradation profile, which support the native tissue and accommodate the healing process, respectively. During the degradation process, any by-products must be noncytotoxic and produce no adverse immune response.

Biodegradable polymers used for tissue engineering scaffolds can be grouped into two main categories: natural and synthetic. Natural biodegradable polymers are typically derived from proteins, polysaccharides, or nucleic acids and can be from plant, animal, or human origin (see Chapter 5, Extracellular

**FIGURE 7.7**

Light microscopy pictures of macroporous CaPs implanted in goat's femoral condyle (magnification: $2\times$). *Courtesy of Dr. Huipin Yuan.*

matrix as a bioscaffold for tissue engineering). Synthetic biodegradable polymers can be tailored to meet specific mechanical, biological, and degradation requirements of the tissue engineered scaffold. They have consistently reproducible properties, are inexpensive to produce in bulk, and are compatible with a wide range of processing techniques (see Chapter 6, Synthetic biomaterials). The processability of synthetic biodegradable polymers allows them to be fabricated into complex three-dimensional architectures for tissue engineering such as fiber networks and porous scaffolds (see Chapter 11, Scaffold design and fabrication). Due to these advantages, much of the research and clinical application of degradable polymers in tissue engineering have been focused on hybrid cell-scaffold constructs using synthetic degradable polymers.

7.4.2 Mechanisms of polymer degradation and erosion

Polymers are large macromolecules composed of repeating chemical units called monomers covalently bound together in a chain. Chain length and length distribution determine polymer molecular weight. Degradation occurs when the bonds between these units are cleaved in a process known as **chain scission**, leading to an irreversible change in material structure and characterized by a loss of properties or fragmentation.¹⁶ Degradation causes molecular weight to decrease as the initial polymer chains are broken down into oligomers and monomers and is accompanied by an eventual loss in mechanical stability. Biodegradable polymers can degrade through a variety of mechanisms including thermal, photo, mechanical, and chemical degradation. For synthetic bioresorbable polymers, chemical degradation through hydrolysis is the primary degradation mechanism of interest in vivo. Hydrolysis is the process by which vulnerable bonds in a polymer chain react with water molecules and break, resulting in shorter polymer chains and eventual polymer degradation. Bonds that undergo passive **hydrolytic degradation** include anhydride, ortho-ester, ester, carbonate, and amide bonds.

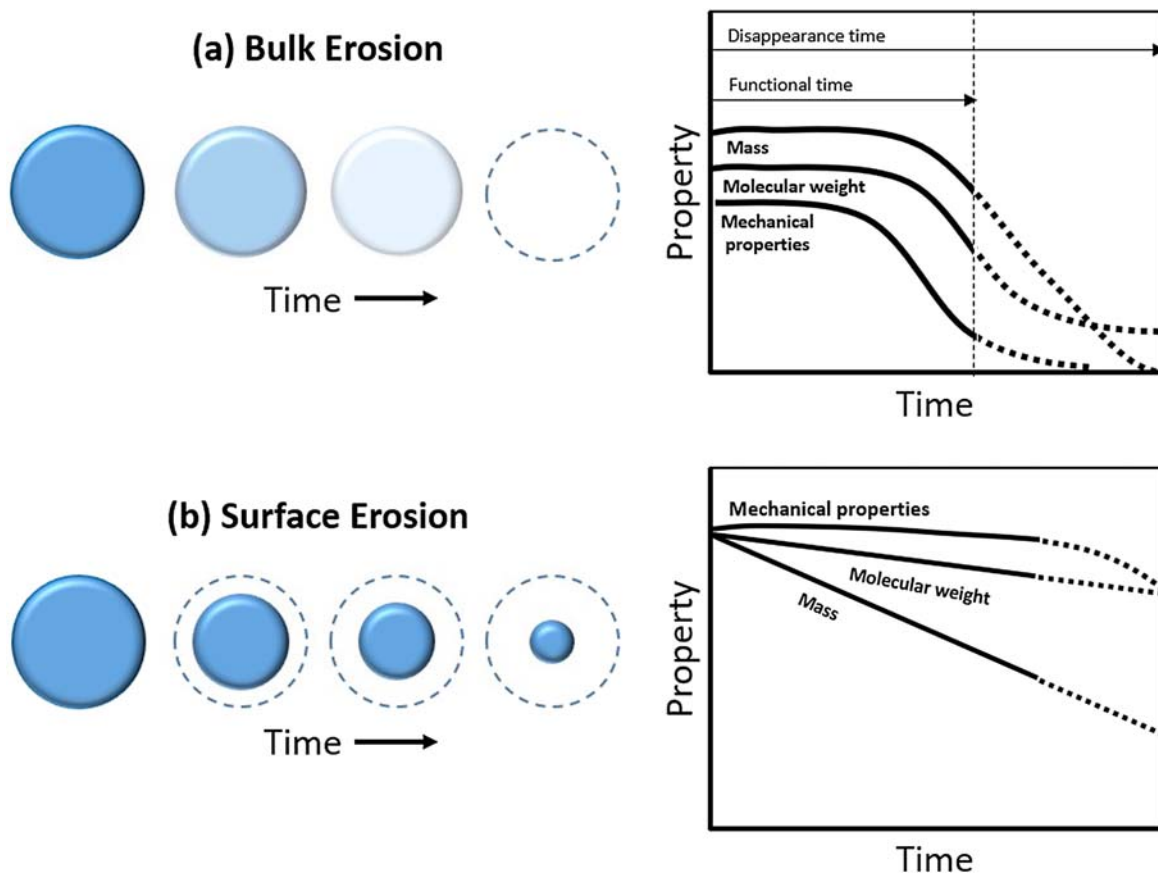
As polymer degradation progresses, it is accompanied by erosion, the loss of mass as oligomers and monomers leaves the polymer, resulting in a change to the physical size or shape of a device. Polymer erosion can be classified as surface or bulk. Whether a polymer undergoes bulk or surface erosion is dependent on two processes: The diffusion of water into the polymer bulk and the degradation rate of the polymer backbone. If the rate of water diffusion is faster than the rate of degradation of the polymer backbone, the polymer will undergo bulk erosion. Conversely, if the rate of degradation of the polymer backbone is faster than the rate of water diffusion, hydrolysis of bonds at the polymer surface will prevent diffusion into the bulk and the polymer will undergo surface erosion. Surface and bulk erosion are schematically depicted in Fig. 7.8.

7.4.3 Bulk erosion

Overview

In bulk erosion, mass loss occurs throughout the material following polymer hydration; water penetrates the polymer at a faster rate than it is broken down.¹⁷ Decrease in molecular weight and loss of mechanical properties begin at the start of degradation, while mass loss is delayed. The external dimensions of the polymer remain essentially unchanged, until disintegration occurs at a critical time point.¹⁸ Most biodegradable polymers that are currently available degrade by a bulk erosion process that predominantly involves simple hydrolysis of main chain bonds, as surface erosion is difficult to achieve.^{19,20}

First, the polymer becomes hydrated and chemical bonds are broken. Next, cleavage of bonds present in the amorphous domains of the polymer causes a decrease in molecular weight and polymer chains are broken into smaller oligomers. Initially, this decrease in molecular weight will not affect the mechanical properties of the device as physical cross-linkages due to entanglements and regions of crystallinity maintain the structure of the material. As molecular weight continues to decrease, polymer chains have a greater degree of movement, which leads to a reduction in mechanical properties. Mass loss begins to occur when the degradation products become soluble in water. Low-molecular-weight chain fragments dissolve and are released into the surrounding medium. The polymer continues to lose mass and physical integrity, and this progresses until there is no remaining material.²¹

**FIGURE 7.8**

Representation of the bulk and surface erosion processes of biodegradable polymers.

Depending on material thickness, geometry, and flow conditions at the implantation site, a phenomenon known as **autocatalysis** can occur, affecting bulk erosion rate and causing the material to degrade in a heterogeneous manner. If thickness is sufficiently low and hydrolysis products are rapidly diffused away throughout the polymer matrix, there will be a gradual drop in molecular weight throughout the sample until a critical value is reached and erosion begins to occur. If degradation products are not rapidly cleared from the polymer matrix, autocatalysis will occur. As degradation products form, those near the surface can be more easily eliminated from the polymer. Toward the center of the material degradation, products are unable to diffuse away, resulting in a localized acidic environment. Since some of the degradation products can be acidic in nature, the increased acidity induces accelerated degradation at the center of the material. The surface of the polymer continues to degrade at its original rate, resulting in a surface-to-center differentiation of mechanical properties and molecular weight.²²

Bulk erosion kinetics of synthetic polyesters such as poly-lactic acid (PLA), poly-glycolic acid (PGA), and their copolymers have been most widely characterized due to their prevalence as biodegradable polymers. These materials predominantly degrade through random chain scission of the ester bond and the hydrolysis of these bonds can be expressed as (Eq. 7.1):

$$\frac{dc_{\text{end}}}{dt} = k' c_{\text{ester}} c_w \quad (7.1)$$

where c_{end} is the concentration of end groups, c_{ester} is the concentration of ester bonds, c_w is the concentration of water, k' is the rate constant, and t is time. For the approximation of bulk degradation, the concentration of water is assumed to be constant throughout the sample over time and the concentration of ester bonds is assumed constant. Integrating the previous equation gives (Eq. 7.2):

$$c_{\text{end}} - c_{\text{end},0} = kt \quad (7.2)$$

where $c_{\text{end},0}$ is the concentration of end groups at time zero, and k is a rate constant equal to $k' c_{\text{ester}} c_w$. A substitution can now be made to account for c_{end} being equal to the reciprocal of number average molecular weight, M_n , and the previous equation becomes (Eq. 7.3):

$$\frac{1}{M_n} - \frac{1}{M_{n,0}} = kt \quad (7.3)$$

where $M_{n,0}$ is the initial number average molecular weight at time zero. This equation can be applied by plotting M_n against time to obtain a linear plot with a slope equal to the rate constant.

To account for the occurrence of autocatalysis, a second equation is used. The kinetics of autocatalysis can be described using the following equation (Eq. 7.4):

$$\frac{dc_{\text{end}}}{dt} = k' c_{\text{ester}} c_w c_{\text{end}} \quad (7.4)$$

Making the same assumptions that c_{ester} and c_w are constant and $c_{\text{end}} = 1/M_n$ the equation becomes (Eq. 7.5):

$$\frac{dc_{\text{end}}}{dt} = k c_w c_{\text{end}} \quad (7.5)$$

From this it can be shown that change in number average molecular weight over time should follow (Eq. 7.6a):

$$M_n = M_{n,0} e^{-kt} \quad (7.6a)$$

This equation is also applied by plotting $\ln M_n$ against time to get a linear plot with a slope equal to $-k$.²³

Molecular weight has a direct influence on polymer strength. As a basic guide, the following relationship has been suggested (Eq. 7.6b):

$$\sigma = \sigma_{\infty} - \frac{B}{M_n} \quad (7.6b)$$

Where σ is the fracture strength, σ_∞ the fracture strength at infinite molecular weight, and B is a constant. Combining Eqs. (7.6a) and (7.6b) produces the expression (Eq. 7.6c):

$$\sigma = \sigma_\infty - \frac{B}{M_{n,0}e^{-kt}} \quad (7.6c)$$

This expression can be used to approximate material tensile strength over time.²⁴

Surface erosion

In surface erosion, degradation reactions are limited to the surface of the polymer material or occur at a significantly higher rate relative to diffusion rate of water into the bulk. Surface-eroding polymers have very hydrolytically labile (easily broken) bonds in their main chains, which react with water rapidly. This makes surface erosion proceed via an erosion front and as a result, the size and mass of surface eroding polymers decrease over time, while molecular weight and mechanical properties of the residual polymer remain unchanged. Since degradation products are concentrated at the surface, they can quickly diffuse away and therefore have no autocatalytic effect on degradation. In surface erosion, the rate of mass loss is proportional to polymer surface area. The predictability of this erosion process makes surface eroding polymers desirable for drug delivery applications, as drug release rate is directly related to the rate of polymer erosion and can easily be altered by changing device geometry. Only a few types of biodegradable polymers such as polyanhydrides and poly (ortho esters) show surface erosion characteristics.^{25,26}

The linear relationship between mass loss and polymer surface area allows for **near-zero-order kinetics** to be achieved and the surface erosion of a disk of material to be expressed as (Eq. 7.7):

$$l = l_0 - kt \quad (7.7)$$

where l is the dimension of the material in the direction of the degradation front at a given time t , l_0 is the original dimension, and k is the kinetic rate constant of degradation.²⁷

Drug release from a device undergoing surface erosion can be approximated using the following relationship (Eq. 7.8):

$$\frac{M_t}{M_\infty} = 1 - \left[1 - \frac{k_0 t}{C_0 a_0} \right]^n \quad (7.8)$$

where M_t is the amount of drug released from the device in time t , M_∞ is the amount of drug present in the device in total, and k_0 is the erosion rate constant. C_0 is the uniform initial concentration of drug in the bulk polymer matrix, and a_0 is the initial radius for a sphere or cylinder or half the thickness of a slab. For a sphere $n = 3$, a cylinder $n = 2$, and a slab $n = 1$.²⁸

Degradation kinetics

Bulk enzymatic degradation of synthetic polymers is commonly predicted using the Michaelis–Menten model of enzyme kinetics (Eq. 7.9):

$$V_0 = V_{\max} \frac{[S]}{[S] + K_M} \quad (7.9)$$

where V_0 is the initial degradation reaction rate, $V_{\max}$ is the maximum degradation reaction rate, $[S]$ is the degradable polymer substrate concentration, and K_M is the Michaelis constant, which represents the substrate concentration required for significant catalysis to occur.

$V_{\max}$ can also be expressed as (Eq. 7.10):

$$V_{\max} = k_{\text{cat}}[E] \tag{7.10}$$

where K_{cat} is the catalytic constant, which describes rate of degradation of the polymer bonds, and $[E]$ is the concentration of enzymatic catalytic sites. These equations give a simple estimation of enzymatic polymer bond cleavage but do not provide information regarding mass loss.²⁹

7.4.4 Factors that influence degradation

How an implant degrades and performs throughout its lifetime is dependent on many factors. These can be grouped into the main categories of polymer composition, molecular weight, morphology, processing, and implantation conditions in vivo. A more comprehensive list of factors that can affect degradation can be found in Table 7.2. Some of these factors can be controlled, while others are inherent to

Table 7.2 Factors that can influence biodegradable polymer degradation rate in vitro and in vivo.

Polymer composition
Chemical structure
Chemical composition
Distribution of repeat units
Presence of ionic groups
Presence of unexpected units or chain defects
Configurational structure
Molecular weight
Polydispersity
Presence of low molecular weight compounds (monomers, oligomers, solvents, initiators, drugs, etc.)
Morphology
Crystallinity (amorphous vs. semicrystalline)
Processing
Processing technique
Annealing
Sterilization technique
Storage history
Implantation site
Adsorbed and absorbed compounds (water, lipids, ions, etc.)
pH
Size and shape
Applied stress
Mechanism of hydrolysis

material processing or implantation environment, and many have an interdependent relationship. By varying the parameters that can be controlled and working to account for the effects of those that cannot, the degradation rate and mechanical properties of the material can be characterized and predicted. This data can then be used to select and tailor material performance to a specific application.

7.4.5 Material composition

At the molecular level, degradation rate is dependent on the accessibility of water to the hydrolytically susceptible bonds. This is directly related to the type of bond charge, the presence of conjugate structures, and any steric effects of chemical side groups.^{26,30} The structures of commonly used synthetic biodegradable polymers can be found in Fig. 7.9.

7.4.6 Bulk eroding polymers

The most widely researched synthetic biodegradable polymers for scaffold applications are poly (α-hydroxy) esters such as PLA, PGA, and their copolymers. This category of biodegradable polymers undergoes bulk hydrolytic degradation at the ester bond in the polymer backbone.

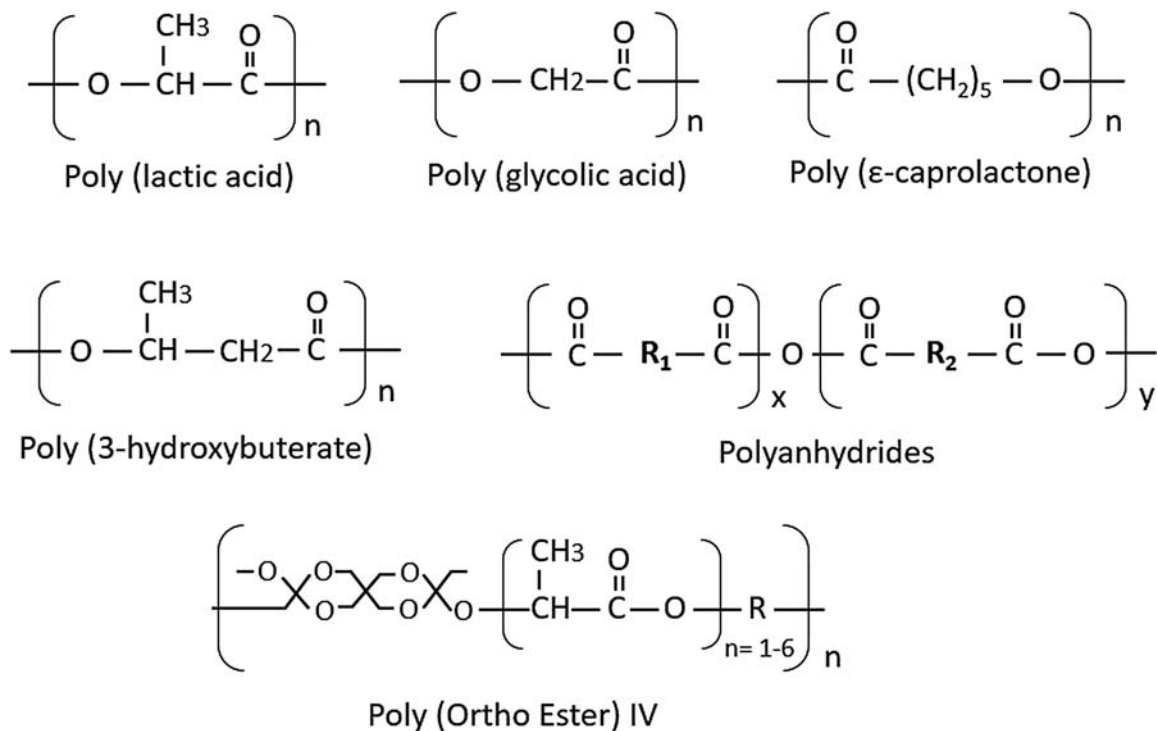


FIGURE 7.9

Chemical structures of commonly used bioabsorbable polymers.

PLA is an aliphatic polymer that exists in two stereo isomeric forms, poly(l-lactic) acid (PLLA) and poly(d-lactic) acid (PDLA), which are dictated by the orientation of the methyl group to the main chain. PLA can also exist in the racemic form, poly (dl-lactic) acid (PDLLA). PLLA and PDLLA are the forms of PLA used for medical device applications. PLLA is semicrystalline (~30%) and has a degradation time ranging from 12 months to 2–3 years. It is used for applications where mechanical properties are important. PDLLA is amorphous and is used in devices not subjected to high loads and in drug delivery applications.²⁷

PGA is the simplest linear aliphatic polyester and has a higher degree of crystallinity than PLLA (30%–55%). By itself, PGA is more hydrophilic than PLA due to the lack of methyl group and has a rapid degradation rate of 6–12 months. Due to its rapid degradation rate, pure PGA is used as in particle or fiber form as a filler material blended with other biomaterials or as short-term tissue engineering scaffolds. PGA is commonly copolymerized with PLA to form poly(l-co-glycolic) acid (PLGA) to tailor degradation and improve material properties.¹⁷ The improved degradation properties of PLGA allow it to be widely used for drug delivery devices, sutures, and longer-term tissue engineering scaffolds.³¹

This family of polyesters also includes poly (ε-caprolactone) (PCL). PCL is semicrystalline and has a slow degradation rate due to the high ratio of hydrophobic methylene groups to methyl groups in the main chain. By itself, PCL has a degradation rate of 2–5 years, which has allowed it to be used in long-term implants and controlled drug release applications. It is commonly used as a copolymer with PLA or PGA for applications where a shorter degradation time is required. PCL has been used for tissue engineering of bone and cartilage (see Chapter 16, Cartilage and bone regeneration).³²

Poly-hydroxybutyrates (PHB) [poly(3-hydroxybutyrate) (P3HB) and poly(4-hydroxybutyrate) (P4HB)] are resorbable polyesters derived from microorganisms instead of chemical synthesis. Although less widely investigated compared to other polyesters such as PLLA and PLGA, this family of polymers has been increasingly researched and used for several medical implant applications such as suture and surgical mesh.^{33,34} PHB is also a popular material for fabricating bone scaffolds or grafts and has been used for wound healing, renal and cardiovascular tissue engineering.³⁵

7.4.7 Surface-eroding polymers

Of the main synthetic biodegradable polymers, polyanhydrides have one of the fastest rates of degradation. Polyanhydrides are typically composed of hydrophobic diacid monomer units connected by anhydride bonds. The anhydride bond is highly labile, and as a result, polyanhydrides have some of the fastest degradation rates of synthetic biodegradable polymers. The combination of the hydrophobic diacid monomers with the rapid hydrolysis rate of the anhydride bond results in a polymer with a reactive surface erosion profile, making polyanhydrides ideal for drug delivery applications.^{36,37}

Poly (ortho esters) are the other main type of synthetic biodegradable polymer that undergoes surface erosion. They are more hydrophobic than polyanhydrides and have a slower and more stable surface erosion profile.³⁸ They are typically synthesized by combining diols with diketene acetals and contain ortho ester groups along the main chain. The development of poly (ortho esters) has evolved through four families, and the current iteration, POE IV, has shown to be most promising for practical applications, as degradation rate and mechanical properties can be accurately and reproducibly controlled

through the incorporation of short glycolic or lactic acid segments into the backbone. Like other surface erodible polymers, poly (ortho esters) have been used for various drug delivery applications.³⁹

7.4.8 Molecular weight

Degradation rate is also dependent on molecular weight and molecular weight distribution. As polymer chain length increases, accessibility of functional groups to water or other degrading species decreases. High-molecular-weight polymers degrade more slowly than low-molecular-weight polymers. A high starting molecular weight is often required to obtain required mechanical properties in many medical device applications. Medical-grade varieties of polymers such as PLA, PGA, and PCL are synthesized using ring-opening polymerization to achieve the necessary starting high molecular weight and a uniform molecular weight distribution.

7.4.9 Crystallinity

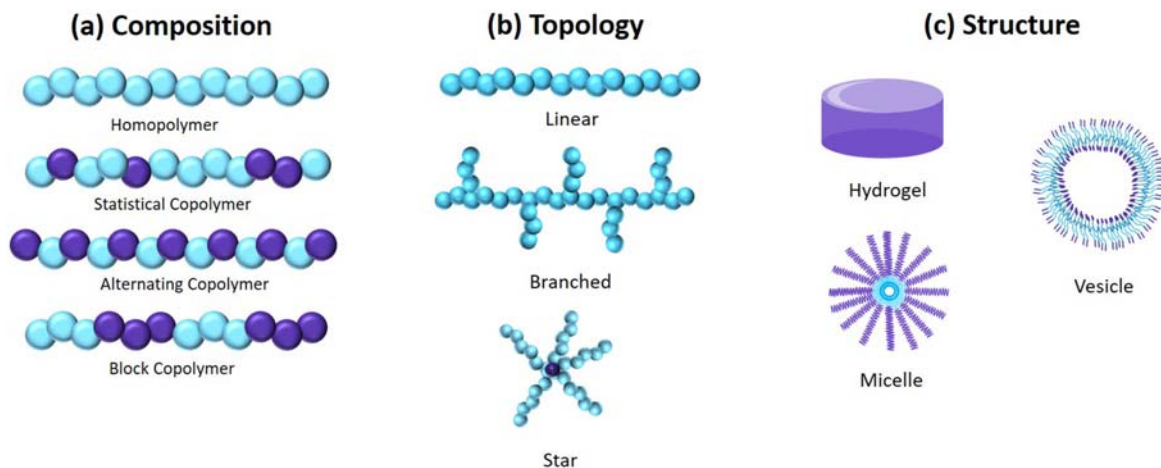
Solid polymers consist of ordered crystalline regions interspersed with disordered amorphous regions. Biodegradable polymers can be amorphous or semicrystalline, and degree of crystallinity is another factor that influences a polymer's degradation rate. Degree of crystallinity is determined by polymer chain ability to pack (determined by chain flexibility and size of functional groups) and the magnitude of intermolecular forces (determined by polarity of functional groups) between the polymer chains. Water diffuses into amorphous regions first, as they are less ordered and less densely packed than the crystalline regions. As degradation occurs, oligomers and monomers can be more easily transported out of amorphous regions.

7.4.10 Glass transition temperature

In addition to crystallinity, another important polymer property with respect to degradation behavior is **glass transition temperature** (T_g). T_g is the temperature at which increased polymer chain mobility results in significant changes in thermal and mechanical properties and represents a transition of the polymer from a "glassy" to a "rubbery" state upon heating. T_g is dependent on polymer chain mobility. Polymer chain mobility is a function of molecular weight, chain flexibility, and presence of functional groups. Less mobile polymer chains will require a higher activation energy before they are able to achieve cooperative movement and as a result have a higher T_g . When polymers are used at temperatures above their T_g , the mobile chains enable more rapid diffusion of degrading species into the bulk polymer, resulting in increased degradation rates. T_g must be taken into consideration when selecting a polymer for in vivo use, as a polymer with a T_g at or near body temperature may experience changes in degradation behavior.

7.4.11 Architecture

Beyond polymer backbone chemistry, functional groups, and molecular weight, another strategy for controlling degradation rate is altering polymer architecture. Composition, topography, and structure can be tailored during polymerization using a variety of polymerization techniques (Fig. 7.10). Copolymers can be designed with statistical-, alternating-, block-, or graft compositions as well as graft, branched, or star topologies. By selecting the frequency, order, and composition of the polymer structure, a material with strategically designed hydrophilicity or crystallinity can be created. For example, a

**FIGURE 7.10**

Various aspects of polymer architecture which can be altered to control polymer degradation rate.

block copolymer with a high ratio of hydrophilic monomers would have an accelerated degradation rate. Copolymers can also be designed to assemble into structures such as micelles, vesicles, or hydrogels through self-assembly or using external stimuli such as pH or temperature.⁴⁰ In addition to being used for the modification of degradation rate, complex polymer architectures can be leveraged for drug delivery applications.

7.4.12 Processing

The methods by which a device is fabricated should also be taken into consideration when determining how it will degrade and perform over its lifetime. Synthetic biodegradable polymers are often fabricated into a useable form with melt-processing techniques such as extrusion, molding, or fiber spinning (see Chapter 11, Scaffold design and fabrication). These types of processing often involve the application of heat and mechanical shear to the biodegradable polymer being used, which can result in thermal and mechanical degradation. **Thermal degradation** causes depolymerization or polymer-chain fragmentation, which results in a reduction in mass and molecular weight. **Mechanical degradation** can cause physical changes such as crystallization, flow, or molecular orientation, or chemical changes such as scission or cross-linking.⁴¹

7.4.13 In vivo degradation

As discussed in the previous section, there are a number of characteristics that can be controlled during polymer synthesis and device design to achieve desired degradation properties. However, what ultimately determines implant degradation and performance is a complex interplay between these factors and conditions at the in vivo implantation site. Hydrolytic degradation is still the primary mechanism of biodegradable polymer degradation in vivo; however, the rate at which hydrolysis occurs can also be influenced by factors such as pH, salts, and enzymes.⁴² Device size and topography can also influence degradation rate, and the location of the implantation site itself determines what the polymer will be

exposed to.¹⁹ In general, in vivo degradation is faster than in vitro due to this added biological response.

Conditions at implantation site

Anions and cations produced by salts present in body fluids such as plasma, interstitial fluid, and cellular fluid can act as catalysts to hydrolytic degradation of polymer bonds. Salt solubility in polymers is related to polymer hydration rate. Hydrophobic polymers do not absorb salts as readily as hydrophilic polymers. The more hydrophilic a polymer is, the greater catalytic effect salts can have on degradation rate. Common ions include sodium, potassium, chloride, and phosphate.⁴³

Another chemical factor that influences degradation in vivo is pH. Depending on implantation site, the pH of body fluid can vary from highly acidic to slightly basic. Biodegradable polymers degrade faster at basic pH due to rate of water absorption over osmotic pressure gradients. At neutral or basic pH, water absorption is increased due to the osmotic gradient created by acidic degradation groups. Another pH-related degradation effect is autocatalysis. If the implantation site does not have enough flow to clear degradation products from the polymer matrix, a localized acidic environment will be created at the center of the polymer, resulting in accelerated degradation in this region.⁴²

In vivo degradation can be further accelerated by mechanical loading at the implantation site. The most common types of mechanical loading in vivo are tensile and compressive, and these are often cyclic in nature. Degradation rate is influenced by type as well as magnitude of load. Loaded polymers degrade faster than unloaded ones, and applying a dynamic load has a greater influence on accelerating degradation rate. Fluid shear is another type of mechanical loading generated by fluid flow at the implantation site. A material located in a site with higher rates of fluid flow will have decreased degradation rate due to the mass transportation effect enabling degradation products to be removed more efficiently.⁴⁴

Inflammatory response

When a material is first implanted in vivo, a wound healing response is triggered by the damaged tissue. Wound healing consists of four stages: hemostasis, inflammation, proliferation, and remodeling.⁴⁵

Polymer–host interactions during the inflammation stage have the greatest contribution to material degradation rate and long-term success of wound healing. In addition to responding to physiologically active substances released by damaged cells and tissues, inflammatory immune cells such as macrophages and neutrophils are triggered by the presence of reactive species in polymer degradation products.⁴⁶ Macrophages and neutrophils are phagocytes that contain lysosomal enzymes. They can attack polymers through exocytosis, where enzymes are released externally on to the surface of the material, or through endocytosis, where fragments of the polymer are engulfed by the phagocytes and destroyed internally. At the start of polymer degradation, enzymes generally attack polymers from the surface due to their large size compared to the polymer matrix; however, protrusions, cracks, or defects on the polymer surface can act as additional points of attack.⁴³ Enzymatic involvement in degradation is more significant during later stages as erosion and physical fragmentation of the polymer occur.⁴⁷ In addition to enzymes, phagocytic cells also release **reactive oxygen species** such as superoxide ions

to destroy foreign matter. These can react to generate other harmful oxidizing agents such as hydrogen peroxide and free hydroxyl ions, which increase polymer degradation rate.⁴²

The degree of remodeling and healing that can be achieved is dependent on the long-term **host response** to the material. Enzymatic activity at the surface of the implant peaks at the start of time after implantation, and dependent on polymer degradation, the inflammatory cell population decreases, and fibroblasts can encapsulate the polymer surface. Once this occurs, the polymer is exposed to a small, steady-state concentration of enzymes close to the implant.⁴³ If degradation products cannot be readily cleared, this can result in chronic inflammation, which continues until complete degradation of the polymer occurs. The presence of chronic inflammation can lead to delayed healing, scarring, or excessive fibrous encapsulation, and the initial inflammatory phase must be resolved before successful wound healing can occur.⁴⁸

Size and shape

Other parameters that influence in vivo degradation are the shape and size of the implant. The immune response to a biomaterial depends to a large extent on the shape of the implant. A notable early study showed that circular rods produced the least **foreign body response** compared to cross-sectional geometries such as triangular or pentagonal.⁴⁹ A further study reinforced this finding by observing that implant shape has a strong impact on phagocyte behavior at the tissue–implant interface, and that smooth, well-contoured shapes with no acute angles are more biocompatible.⁵⁰ Higher surface area due to fragmentation or porosity can also affect tissue response.

The size of the implant also plays a role in immune response. Larger implants produce a proportionally higher magnitude of foreign body reaction and fibrosis. For polymers that are susceptible to autocatalysis, in addition to being dependent on flow conditions at the implantation site, there is a critical thickness at which hydrolytic degradation will be accelerated due to accumulation of acidic degradation products in the implant.⁵¹ Conversely, it has also been found that if implant size is too small, an increased immune response can also be observed independent of total implanted surface area. In addition, the influence of geometry or curvature on immune response is also significant at the microscale level.⁵²

Overall, the in vivo degradation rate of a biodegradable polymer is dependent on many implantation site-specific factors, which in turn are influenced by implant characteristics. This complex interaction stresses the importance of performing relevant in vivo experiments in addition to in vitro testing when selecting a biodegradable polymer for a specific tissue engineering application.

7.4.14 In vitro testing and characterization

For in vitro testing of biodegradable polymers, most degradation studies are based on samples incubated at body temperature ($37 \pm 1^\circ\text{C}$) in a phosphate buffered saline solution maintained at $\text{pH } 7.4 \pm 0.2$. These guidelines, as well as further testing and evaluation criteria for in vitro degradation of biodegradable polymers, are outlined in technical standards ASTM F1635-16 and ISO 13781:2017. While ASTM F1635-16 and ISO 13781:2017 create a solid framework for in vitro degradation testing, their primary limitation for practical use lies in degradation time. In many cases, it is desirable to observe a material through advanced stages of degradation; however, for materials such as PLLA or PCL, this can take multiple years when tested at the physiological conditions prescribed by the standards.

There have been a number of approaches to establishing an accelerated test method, including varied media pH, introduction of an applied strain, addition of enzymes to the media, and elevated media temperature.^{53–56} The most widely used approach to induce accelerated degradation is elevated temperature, as its relation to degradation rate is well characterized by the Arrhenius equation. Performing degradation testing at elevated temperatures has been shown to greatly decrease degradation time in various biodegradable polymers. While testing at elevated temperatures is a convenient method for increasing the practicality of carrying out long-term degradation experiments, the influence of elevated temperature on the degradation mechanism occurring must be taken into consideration before using data to predict device lifetime in vivo.

In addition to the testing outlined in the standards, further characterization techniques can be used to evaluate material degradation beyond change in molecular weight and mechanical properties such as differential scanning calorimetry (DSC), scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR).

7.4.15 In vivo testing and characterization

Once degradation behavior has been thoroughly characterized using in vitro testing, in vivo degradation studies are used to observe device performance in an animal model. In vivo degradation assessments should be performed within the guidelines of ISO 10993.⁵⁷ In vivo degradation testing is typically conducted by implanting the polymer of interest in an appropriate animal model and retrieving it for characterization at set time points.

The immune response can be studied by sectioning and staining the explanted polymer and surrounding tissue to undergo histological evaluation. The presence of degradation-related enzymes such as lysosomes can be monitored to evaluate their role in the degradation process. Advanced imaging techniques such as magnetic resonance imaging (MRI) or microcomputed tomography (micro-CT) can also be used to track morphological changes of the scaffold caused by degradation in vivo.³⁵ Like in vitro testing, mass loss can also be used to determine degree of degradation. This measurement can be difficult to carry out on materials that have reached advanced stages of degradation, and it is often used at early time points.

7.5 Biodegradable metals

All metals corrode to a certain extent when provided with the right environment; however, some are significantly more reactive than others. Most metal implants in clinical use today are permanent and intended to remain in the body for the lifetime of the patient, or until they are surgically removed. However, there is increasing interest in the development of implants made from fully biodegradable metals, particularly for bone tissue and load-bearing applications.

Biodegradable metals are defined by Liu et al., (2019)⁶² as metals which are “expected to corrode gradually in vivo, with an appropriate host response elicited by released corrosion products, which may pass through, be metabolized or assimilated by cells and/or tissue, and then dissolve completely upon fulfilling the mission to assist with tissue healing with no implant residues.”

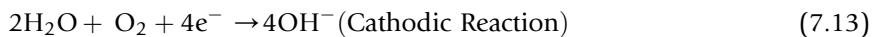
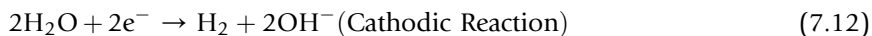
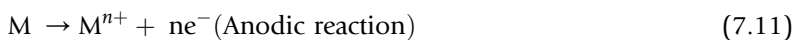
This definition outlines some of the criteria required to classify a metal as a suitable biodegradable material for medical applications, thus setting them apart from existing permanent metal implants in current use. Biodegradable metals offer potential advantages over other biodegradable materials due to their high strength-to-weight ratio (specific strength), which can adequately provide mechanical support to the healing tissue until sufficient regeneration has taken place and a gradual load transfer back to the healed bone is facilitated.^{27,58}

Development of biodegradable materials must take into consideration that the corrosion rate is an appropriate match to the healing time in order to sustain the necessary mechanical support needed by the injured tissue. The physiological response to corrosion products and the mechanisms by which these corrosion products are excreted from the body are also important factors in the design of biodegradable metals.

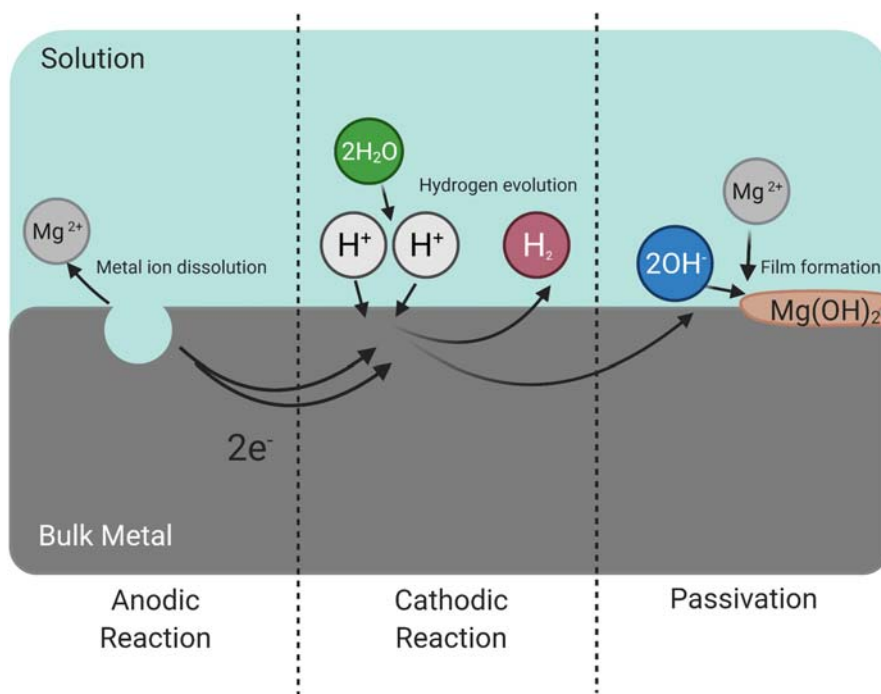
Biodegradable metals will be discussed in the context of these design features, including the corrosion process, methods of controlling corrosion rates, and methods of evaluating corrosion both in vitro and in vivo. Progress in the development of the most researched biodegradable metals for tissue engineering will be discussed along with the remaining challenges inhibiting their widespread clinical application.

7.5.1 Principles of metal corrosion

Corrosion occurs by coupled electrochemical half-cell reactions. Metal loss occurs as an anodic reaction where electrons are lost from the bulk metal, and there is dissolution of the metal ions as shown in Eq. (7.11). In an anodic reaction, there is an increase in the metal species' oxidation number, and it is therefore known as an oxidation reaction. In the cathodic reaction, water and dissolved oxygen are reduced to hydroxyl ions when the metal is exposed to neutral or basic solutions as shown in Eqs. (7.12) and (7.13).⁵⁹ In a cathodic reaction, there is a decrease in the oxidation number of the given species, and this is called a **reduction reaction**. The combined anodic and cathodic reaction products lead to the precipitation of an oxide layer on the metal surface, which can provide a small amount of protection to the bulk metal from the environment as shown in Eq. (7.14).^{60,61} The corrosion process of pure magnesium is illustrated in Fig. 7.11. From the left to right, there is dissolution of the magnesium ions in the anodic reaction. Two electrons are then taken up in the cathodic reaction, and water is converted into hydrogen and hydroxyl ions. During passivation, the dissolved magnesium reacts with the hydroxyl ions to form a precipitate layer of magnesium hydroxide on the metal surface.



Metals will exhibit different corrosion potentials in different environments; however, the standard electrode potential provides information on the tendency of a metal to degrade in a neutral aqueous

**FIGURE 7.11**

An example of the corrosion process of pure magnesium in a neutral solution. Created with biorender.com.

environment. Any metal with a **standard electrode potential** lower than that of hydrogen ($E^0 = 0 \text{ V}$) has the potential to degrade under standard conditions in water. The standard electrode potential is the relationship between a metal's electric energy and its **Gibb's free energy**. The lower the Gibb's free energy change of a metal in a reaction, the lower the electrode potential and therefore, the more readily the metal will undergo spontaneous corrosion.^{60,62} The most common metals under investigation as biodegradable implant materials are magnesium, iron, and zinc alloys.⁵⁸ These alloys have standard electrode potentials of $\text{Mg} = -2.37 \text{ V}$, $\text{Zn} = -0.763 \text{ V}$, and $\text{Fe} = -0.440 \text{ V}$. The corrosion rates of these metals decrease in reactivity from magnesium to zinc to iron.²⁷

Corrosion in the in vivo environment

The in vivo environment differs greatly from a neutral aqueous solution at standard conditions and factors such as pH, temperature, and fluid dynamics all play a critical role in the biodegradability of metals in the human body.

When a metal is first introduced to a corrosive environment such as body fluids, corrosion rates are initially high before the formation of the passive oxide layer on the outer surface. The development of the thin oxide layer on the outer surface of the metal implant causes the corrosion rate to decrease by several orders of magnitude. The extent to which this oxide layer can protect the bulk metal implant

from the aggressive biological environment depends on the stability of the film and the rate of ion transfer through it.⁶¹

From a material perspective, the rate of ion transfer through the film is influenced by the composition of the oxide film, its structure, and thickness as well as the presence of defects on the material surface. From a biological perspective, the electrolyte composition, temperature, and exposure time all play a role in determining the extent of protection afforded by the oxide layer.

Blood plasma contains a high concentration of chloride ions, which are an aggressive oxidizing agent, and one of the most reactive in the halogen series of elements. This can induce localized corrosion at defect points and has detrimental effects on the mechanical integrity of an implant. Furthermore, the body temperature of 37°C can accelerate degradation reactions or in certain metals, it may change the mechanism of corrosion from that which would typically occur at room temperature.⁶¹

The presence of proteins in the physiological environment can also change the mechanism and the rate of corrosion of a metal through the formation of biofilms. This can impact the oxygen content surrounding the implant, which, depending on the metal, and the corrosion mechanism, may slow corrosion reactions or conversely, leading to preferential corrosion in oxygen-depleted regions. Different implant sites also have varied hemodynamic conditions, which impact mass transfer of degradation products from the surface of the metal implant and result in changes to the corrosion reactions.

Surface finishes, geometry, and design of an implant can also influence the degradation rates. These effects are particularly important in the development of bone scaffolds and will be discussed further in subsequent sections.

Localized corrosion effects

Localized corrosion processes can cause rapid dissolution of the bulk metal leading to embrittlement of the material and early mechanical failure at stresses well below the materials yield strength. Localized corrosion in vivo typically occurs at sites where there are material inhomogeneities or changes in the local environment.⁶¹ Localized corrosion effects complicate the corrosion process of implants. It causes nonuniform degradation, resulting in early mechanical failure, and the apparent random nature of the breakdown can make accurate prediction of implant performance a challenge. Some of the common localized corrosion effects are pitting corrosion and microgalvanic corrosion, while specific loading conditions of the implant and environmental conditions associated with the implant site may lead to corrosion fatigue, stress corrosion cracking, or crevice corrosion.

7.5.2 Magnesium-based implants

Magnesium alloys are one of the more extensively studied biodegradable metals for bone tissue engineering applications. While pure iron shows promise due to its favorable mechanical properties such as its high elastic modulus and good biocompatibility, its degradation rates are too slow to appropriately match tissue repair. Significant modifications are needed to increase its degradation rate to enable its use for medical implant applications. Conversely, zinc and its alloys demonstrate favorable degradation rates, which are more appropriate even compared to magnesium and its alloys. However, its poor strength and ductility limit its use in load bearing applications such as bone scaffolds or orthopedic

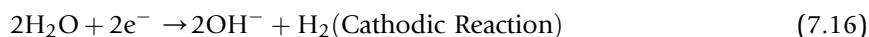
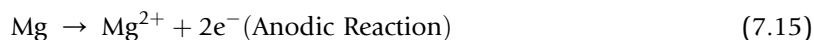
screws and pins.⁶³ While there are several studies that have examined Zinc alloys for bone scaffold applications,^{57,64–66} the development of zinc based implants is limited to the research context.²⁷ Magnesium-based alloys have had some success in the clinical setting with a number of regulatory approved implants, including Magmaris (Biotronik AG, Bülach, Switzerland) cardiovascular stent,⁶⁷ Magnezix (Syntellix AG, Hannover, Germany) compression screws,⁶⁸ and Resomet (U & I Corporation, Seoul, Republic of Korea) K-wire and pins, headless screws and cortex screws.⁶⁹ Recent advances in additive manufacture techniques have driven developments in magnesium-based bone scaffolds, which can be customized for patient-specific geometries.⁶³

One of the greatest challenges to the clinical translation of magnesium-based implants has been the rapid degradation rates observed, which can lead to early implant failure and hypomineralization of the bone tissues due to increased magnesium ion concentration and gas accumulation.^{27,70} As a result of these observed effects, the development of novel alloy materials, as well as surface coatings, has been explored in recent years to stem the degradation rates.⁷¹ These material developments have been studied both in vitro and in vivo to assess their degradation rates and the possible effects on bone tissue regeneration.

The remaining sections will examine magnesium and its alloys in more detail as the most promising biodegradable metal for tissue engineering applications.

Magnesium corrosion

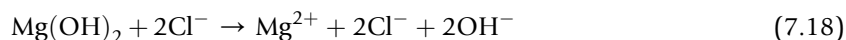
The corrosion of magnesium in an aqueous environment produces magnesium hydroxide and hydrogen gas according to the following anodic and cathodic reactions given in Eqs. 7.15 and 7.16⁷²:



Magnesium ions then react with hydroxyl ions to produce a magnesium hydroxide protective layer on the substrate surface that impedes the corrosion of the bulk material; this occurs according to the following reaction given by Eq. (7.17)⁷²



Magnesium is a very reactive metal and demonstrates poor corrosion resistance in the presence of electrolyte species such as chloride ions or in regions where there are impurities in the metal substrate. Localized corrosion effects can be observed and have a significant effect on the degradation rate of the metal. Pitting corrosion is a common corrosion mechanism for magnesium, while metallic impurities such as the presence of nickel, iron, and copper act as cathodic sites for microgalvanic corrosion to occur.⁷³ In the presence of chloride ions in the physiological environment, the magnesium hydroxide precipitate is converted into soluble magnesium chloride according to Eq. (7.18) and the surface of the metal can undergo pitting corrosion.⁷³



Controlling magnesium degradation rates

One of the primary challenges with the development of magnesium alloys is their rapid degradation rates, particularly in the early stages post implantation. To overcome these issues, a number of design mechanisms can be employed to enhance the performance of these materials. Namely, the composition of the alloy material, the structure of the material, and the surface finish of the material. These all have an influence on the material's degradation profile.

The corrosion behavior, mechanical properties, and biocompatibility are influenced by the alloying elements and the microstructure of the metal substrate.²⁷ Some of the common alloying elements utilized in magnesium-based systems are aluminum (Al), calcium (Ca), lithium (Li), manganese (Mn), zinc (Zn), zirconium (Zr), strontium (Sr), cerium (Ce), erbium (Er), gadolinium (Gd), lanthanum (La), neodymium (Nd), and yttrium (Y).⁷⁴ These elements react with the magnesium substrate and form intermetallic phases, which either precipitate along the grain boundaries or enter solid solution in the magnesium matrix. Elements such as calcium, zinc, zirconium, and strontium only improve the corrosion resistance of magnesium when they are in small concentrations, at higher concentrations, the corrosion rate deteriorates significantly. However, it is reported that the addition of strontium can improve compressive yield strength while calcium can promote osseous growth for orthopedic implants.⁷⁴

Magnesium alloys can undergo substantial galvanic corrosion due to metal impurities or the presence of second phases precipitated along grain boundaries, which act as cathodic sites due to their relative thermodynamic stability.⁶⁰ This results in significant localized corrosion. Methods of grain refinement can alter the density of the grain boundary and the distribution of the intermetallic phases.⁷⁴ Grain refinements due to rapid solidification techniques have shown more uniform corrosion behavior due to the extended solubility limit of the α -Mg matrix resulting in better distribution of the intermetallic phases. Such grain refinements can also be achieved through plastic deformation techniques such as equal channel angular pressing (ECAP), high pressure torsion, or cyclic extrusion and compression.⁶⁰

The addition of alloying elements has a significant influence on the grain size, boundary density, and phase distribution in the metal, which in turn influences the mechanical properties and corrosion behavior. The volume fraction, distribution, and electrical potential of secondary phases have a profound impact on magnesium alloy corrosion.⁷⁴ There are many reports in the literature that aim to study the effects of alloying and processing on the microstructure, mechanical properties, and corrosion behavior of magnesium alloys.

Another method of improving or controlling the corrosion behavior of magnesium alloys is through surface modifications and coatings. Coatings act as a physical barrier between the environment and the bulk metal and allow for the corrosion resistance of the metal surface to be tailored while preserving the properties of the bulk metal. Coatings such as inorganic calcium phosphate are one of the main components of bone tissue and demonstrate good biocompatibility, bioactivity, and bone inductivity. Polymer coatings such as poly-lactic-acid and polyglycolide are often used as drug carriers for steady and controlled drug release to an implant site; however, issues with adhesion of such polymer coatings to the magnesium substrate are a challenge and in some cases hybrid coatings have been employed to improve adhesion properties.

In addition to altering mechanical and corrosion properties of the magnesium alloys, surface modifications and alloying elements also have an impact on the material biocompatibility. Any alloy selected therefore needs to be carefully investigated for local and systemic toxicity effects in the intended implant site.⁶⁰ It is essential that any alloying addition does not exceed the permitted concentration levels and that there is sufficient control of the local metal ion release and efficient removal from the local tissue environment.⁶⁰

In vitro corrosion and test methods to measure magnesium corrosion: One of the principal steps in biodegradable materials development is in vitro assessment of their degradation rates, mechanical properties, and biocompatibility to evaluate the potential efficacy and safety profile prior to progressing to preclinical animal studies. Different physiological testing solutions have been shown to produce not only different corrosion products but also different corrosion rates.⁷⁵ In general, it has been reported that solutions such as SBF and DMEM provide better correlations to in vivo degradation rates.⁷⁶

In addition to the physiological medium used, factors such as temperature, pH, and flow dynamics all impact the corrosion mechanism of magnesium. It is important to employ physiological temperatures as close to 37°C as possible. It has been shown that higher temperatures increase the corrosion rate of magnesium alloys, with reports of a twofold increase in corrosion rate when comparing magnesium tested at atmospheric temperatures of 20°C compared to 37°C.⁷⁶

In the in vivo environment, the pH of the body is maintained at 7.4 through a buffering mechanism in the blood, which consists of carbonic acid and bicarbonate anions. The inclusion of such a buffering system in the physiological fluid used for in vitro testing helps to maintain the pH closer to that of the human body as degradation progresses.^{75,76} This prevents the development of a localized alkaline environment, which facilitates the precipitation of corrosion products on the metal surface.

Flow dynamics impact corrosion by carrying away the corrosion products and preventing passivation. In static conditions, there is a decrease in corrosion rate over time due to the formation of a passive layer, whereas in dynamic conditions, there is not an observed decrease in corrosion rate, since there is no formation of the protective layer.⁷⁶ The intended host site should be considered when deciding flow conditions for simulated in vitro testing. In general, there are a multitude of factors that influence the corrosion rate in vivo and in vitro, and not all of them are fully understood. There remains an inconsistent correlation between in vivo and in vitro corrosion rates.

The simplest methods of estimating corrosion rate are through mass loss measurements. In this process samples are weighed before and after immersion in a physiological fluid for a fixed time. After immersion, the corrosion products are removed by pickling, usually with a chromic acid solution before weighing. The test method is outlined in ASTM G1 and G31, which provide details on experimental setup, apparatus, and sample preparation including removal of corrosion products. Care must be taken when choosing the acid solution for removing corrosion products as some alloying elements will react and can be inadvertently removed along with the corrosion layer, confounding the results. Rare earth containing alloys and alloys containing silicon or zirconium tend to react with chromates, and this test method may not be appropriate for all alloys.

The corrosion rate can be calculated by the following Eq. (7.19) using the mass loss information:

$$C_{\text{rate}} = K \frac{\Delta M}{A t \rho} \quad (7.19)$$

Where C_{rate} is the corrosion rate given in mm/year. $K = 8.76 \times 10^4$ is a conversion factor for the units. ΔM is the change in mass of the sample in grams, A is the exposed surface area in cm^2 , t is the exposure time in hours, and ρ is the material's density in g/cm^3 .⁷⁷

In conjunction with mass loss measurements, another immersion experiment to estimate corrosion rates is the measurement of evolved hydrogen. It is one of the most commonly employed methods of estimating the corrosion rate of magnesium reported in the literature. A simple setup proposed by Ref. 72 is illustrated in Fig. 7.12 and often adapted for physiological conditions. The guiding principle for the use of this experimental method is that for every mole of hydrogen released, a corresponding mole of magnesium is also removed from the bulk metal. However, there are limitations in the interpretation of these measurements in physiological solutions, where there are hydrogen-binding

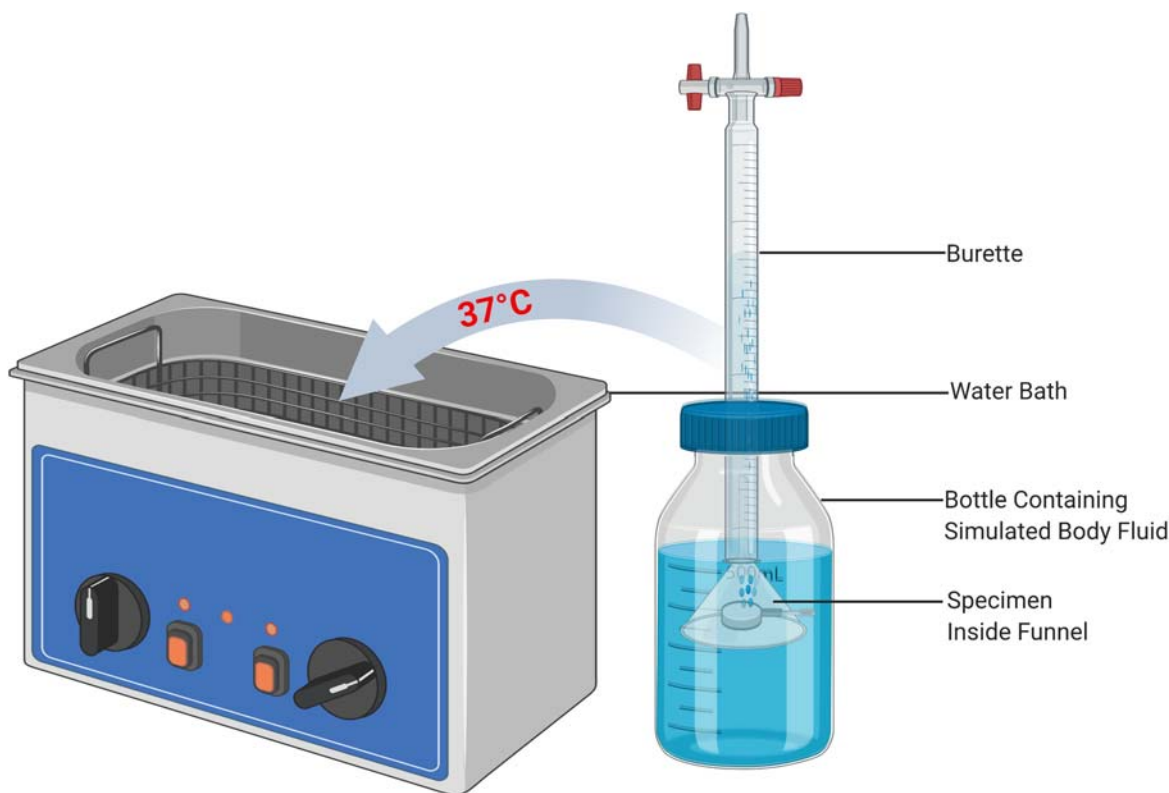


FIGURE 7.12

Schematic example of an experimental setup for the physiological assessment of hydrogen evolution from magnesium alloys. *Created with biorender.com.*

ingredients, which may interfere with the volumes of gas released. It is a good idea therefore to analyze the gas released from such experiments.²⁷ Despite this, hydrogen evolution provides useful information of the corrosion process overtime as it is possible to take measurements over smaller time points when compared to mass loss measurements. It is also valuable in the side-by-side comparison of differing materials.

Electrochemical corrosion testing methods are commonly employed to evaluate the kinetics of the corrosion process. These test methods are well established in corrosion science and have a high degree of reproducibility due to the high degree of control over the test parameters.⁷⁸ Potentiodynamic polarization and impedance measurements are the most common electrochemical test methods employed for biodegradable metals.⁶⁰

The microfocus computed tomography (microCT) is employed as an *in vivo* method to evaluate the corrosion rate of a sample. This allows for estimation of the degradation rate in animal models at intermediate time points without sacrifice of the animal as is the case with *in vivo* mass loss measurements.⁶⁰ MicroCT allows for high-resolution scans that can be stacked to produce a volumetric image of the implant *in situ*. MicroCT also allows for assessment of the bony tissues surrounding an implant⁷¹; however, it is unable to assess composition and structure of bone, which give indications of the strength of the newly formed bone.⁷⁰ This method can also be employed for explanted samples.

Mg-based tissue scaffolds: designing for function and enhanced properties

Magnesium alloys have been employed successfully for orthopedic screws, plates, nails, and wires. However, the development of magnesium-based scaffolds for bone tissue engineering is not as well advanced, with studies predominantly being conducted *in vitro* to optimize material design and properties. The objective of magnesium-based scaffold development is primarily focused on the treatment of **critical sized bone defects**. Critical-sized bone defects are defined as those that will not heal spontaneously within a patient's lifetime; they are often the result of trauma, revision surgery, tumor resection, infection, or osteodegenerative diseases.^{77,79}

The ideal bone scaffold for critical-sized bone defects is defined by Ref. 79 as *"providing an environment that mimics the body's natural healing process. Such a graft would promote osteogenesis and angiogenesis, while having sufficient mechanical strength to promote integration with host tissues and facilitate load transfer under weight-bearing conditions."*

It is proposed that the mechanical properties of magnesium are a better match compared to other metal counterparts or even polymers for bone tissue healing and have the potential to avoid such issues as stress shielding. **Stress shielding** occurs when implants designed to repair fractures or replace joints have a much higher stiffness than bone and leads to a loss of strength in the surrounding bone as a result of the decrease in physiological loading of this bone.⁸⁰

Human cortical bone has a compressive strength of 130–180 MPa while traditional implant materials such as titanium alloys and cobalt chromium have a compressive strength of 778–1117 MPa and 450–1000 MPa, respectively.⁸¹ Magnesium and its alloys have a reported compressive strength in the range of 65–100 MPa and a density of 1.7–2.0 kg/m³, which closely matches that of human cortical bone.⁸¹

Bone scaffolds are designed with a porous structure, which is necessary for tissue ingrowth, the diffusion of nutrients, and prevents the loosening of the implant. However, porous design significantly impacts the compressive strength of the material and the increased surface area causes significant hydrogen release and rapid degradation, which can result in premature implant failure.^{60,77,79} Further to this, while the mechanical properties of magnesium and its alloys are theoretically a good match for supporting bone healing; the mechanical properties of cancellous bone and cortical bone vary significantly and as a result, the mechanical properties of the healing bone tissues change as the healing process progresses.⁷⁹

There are several methods of fabricating porous metal scaffolds, including negative salt pattern molding, titanium wire space holders, powder metallurgy, hydrogen injection, and laser perforation, all of which are outlined by Ref. 82. More recently, laser powder bed fusion has been employed as a method of additive manufacturing of porous magnesium scaffolds, which can be tuned to specific patient geometries.^{77,83}

Magnesium additive manufacture is still in its infancy, and the reactive nature of the metal powder, which can readily react with atmospheric oxygen, poses many challenges during processing. There are a number of recent studies that have developed porous magnesium scaffolds by additive manufacturing methods.^{77,83–86} These authors have examined the impact of the rapid solidification and repeated heat exposure during manufacture on the microstructure.^{77,85} They have also studied the effects of heat treatments and varied pore size and shape as well as strut diameter on the degradation and biocompatibility profile of these scaffolds in addition to the effects of coatings such as microarc oxidation coatings.^{77,83} In addition, the fatigue behavior of such scaffolds has been examined during compression–compression fatigue tests by Ref. 84. Although, progress is still to be made, the materials developed to date show promise as scaffold materials. Optimization of the processing parameters to produce more stable morphologies, together with modification of porous structure design and the use of postprocessing treatments, has the potential to yield magnesium scaffolds with improved mechanical and degradation performance.

7.6 Future perspective

Much has been learned regarding degradation of biomaterials in the past few decades, and there has been significant progress in optimizing biomaterials for scaffold applications. Key to the future success will be ensuring that the degradation process occurs harmoniously with tissue regeneration. This means optimizing degradation rate so that it does not occur before tissue can form a supportive structure and ensuring that degradation products do not negatively interfere with this process.

Beyond choice of biomaterial, it is important to remember that processing method and performance are directly interlinked. This is particularly the case for biodegradable materials, which are especially sensitive to processing conditions. For example, polymers tend to thermally degrade during melt processing, such as fused deposition modeling (FDM), metals oxidize during selective laser sintering (SLS), and bioceramics can change character if low level of impurities is present.

For bioceramics, an exciting prospect is the opportunity to intentionally include bioactive components within a degradable scaffold that influence biodegradation character and are released in a controlled

manner via the bioresorption process. This can include dopant ions in calcium phosphate bioceramics or bioglass, where elements such as strontium, lithium, silicon, and zinc have been shown to play a role in osteogenesis.⁸⁷ Although bioceramics and bioglasses themselves are brittle in nature, this limits their application where reliable mechanical properties are required, there are good prospects anticipated through combining them with metal or polymer matrices to form hybrids with tailorable properties for broader applications. There is also new understanding on the osteoinductive nature of bioceramics, which highlights the fact that in vivo mineralization, to form a bioactive apatite layer is a requirement for osteoinduction.¹²

Current research on degradable polymers in tissue engineering focuses on balancing the ability to support tissue growth with the degradation rate. Despite synthetic biodegradable polymers having many advantages in processing compatibility and property control, their major limitation is in their lack of bioactivity. This has limited the success of tissue engineering scaffold fabricated solely from synthetic polymers. Various strategies to improve bioactivity have been explored, such as creating hybrid scaffolds from synthetic polymers coated or blended with bioactive natural materials. A natural–synthetic composite polymer scaffold can avail of the beneficial characteristics of both materials.

The development of biodegradable metals has come a long way in the past two decades. Like many biodegradable materials, issues associated with control of mechanical properties alongside degradation rates remain a significant challenge for their clinical translation. Alloys of iron, zinc, and magnesium have been explored in the literature for their degradation and biocompatibility potential as implant materials. However, to date magnesium and its alloys have had the most success and continue to show the greatest potential in the clinical setting as implant materials. From a tissue engineering perspective, the advent of additive manufacture may allow for further optimization of metal-based tissue scaffolds. However, there is still a significant amount of work remaining in their design and manufacture, and to date, there is limited in vivo data to provide further insight into their performance. Nevertheless, the research being conducted is gathering momentum, and there is much promise within this field of research.

From a manufacturing perspective, the biomaterial must be suitable for scaling up to a commercial level, it must be compatible with processing techniques to create the final product design, and it must be compatible with a **terminal sterilization** modality or aseptic processing technique.

When studying the degradation behavior of these next-generation materials, in vivo testing used jointly with thorough in vitro characterization can provide a more complete picture of how a biodegradable polymer scaffold will degrade over its lifetime in vivo. A biomaterial can demonstrate biocompatibility over relatively short-term in vitro evaluation, but longer-term tests, ideally up to the point where the biomaterials' scaffold has fully resorbed, are required to ensure confidence in clinical outcomes and avoid any unanticipated negative response to degradation products. Furthermore, there is still the need for the development of new characterization strategies to examine the tissue–material interface relationship in vivo. There is also a need for further development and standardization of test methods to create a replicable knowledge base on which new research can be built. This combination of robust test methods and a better understanding of degradation behavior will enable the further development of biodegradable biomaterials with superior in vivo performance and the potential for widespread application.

Summary

- Bioceramics degrade via mechanisms including: (1) physicochemical dissolution accompanied by possible phase transformation, (2) cellular degradation mediated by multinucleated cells, and (3) mechanical fragmentation due to a loss of structural integrity resulting from mechanisms (1) and (2).
- Factors that included bioceramic degradation rate broadly include chemical reactivity, degree of crystallinity, exposed surface area, and presence of certain additives.
- Degradation generally occurs at the surface, with gradual erosion of the bioceramic and release of soluble ions.
- Polymers generally degrade hydrolytically by either a bulk or surface erosion process, with bulk erosion resulting in loss of mechanical properties ahead of loss of mass.
- Although hydrolytic degradation is the dominant process for polymers, some can undergo enzymic degradation, with surface erosion being more common than bulk for the latter.
- Factors that included polymer degradation include molecular weight, crystallinity, and molecular structure.
- Some polymer degradation products can be acidic in nature, potentially inducing a localized inflammatory response.
- Metals biodegrade via a corrosion process, which can result in formation of oxides, hydroxides, and hydrogen gas.
- Corrosion is a surface erosion process although this can be nonuniform, resulting in surface pitting.
- Magnesium metals have generated most interest in the field of biometals, however, display relatively high corrosion rates in vivo. Iron and zinc alloys also warrant consideration.

Classical experiment

The discovery of bioactive glasses

While attending a US Army Materials Research Conference in 1967, Larry Hench was horrified by the stories of Vietnam War battlefield casualties that were causing thousands of amputations in young soldiers. "Instead of making materials to destroy people," Hench took the challenge to "make materials to repair people." Indeed, in the 1960s, bone implants made from metals and plastics were not suitable for bone repair, as they were rejected too often by the body. His idea was to develop a material that would be accepted by the host bone. Because bone is composed of a mineral phase made up of calcium and phosphate, he hypothesized that an implant containing calcium and phosphate in the right proportions would not be rejected by the body. He designed three compositions of glass that contained calcium and phosphate, silicon dioxide to

hold the structure together, and soda to stimulate the melting of glass in the furnace. These samples were then implanted into the thighbones of rats. Larry Hench was called by the surgeon who was taking care of the animal experiments and reports his conversation:

Six weeks later, Ted called me and yelled "Larry, what is that stuff you gave me?" He was so excited, I thought for sure the glasses had killed the rats. So, I quickly replied, "Calm down, Ted. They're only the first tries. There are lots of other compositions I can make." I'll never forget his answer. He said "Larry, you don't need to make any other glasses. The first one's work. Those implants won't come out. They are bonded to the bone. I've never seen anything like it before." He was right. The glass implants did not come out ... A bond formed that was as strong as

Classical experiment—continued

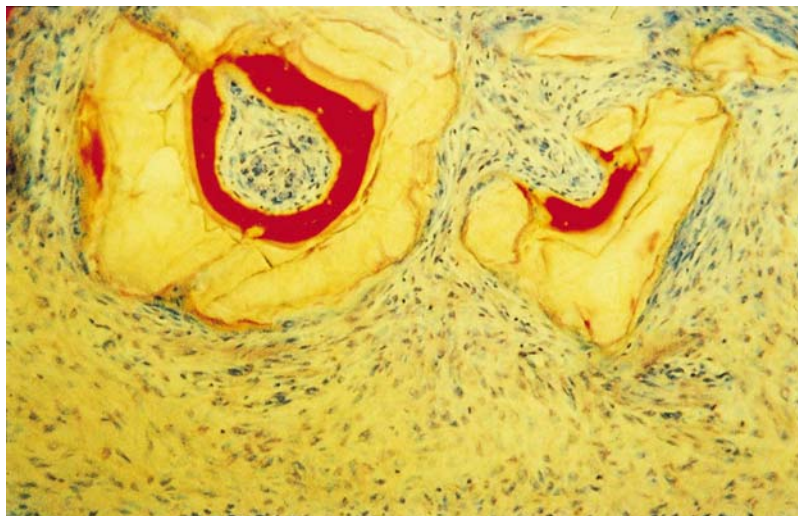


FIGURE 7.13

Bone tissue formation in excavated bioactive glass particles of narrow size range. Bone tissue is stained red. Also note the channels connecting the interior of the particle with the surrounding milieu. From *Bioactive ceramics: The effect of surface reactivity on bone formation and bone cell function*, Ducheyne, P., Qiu, Q., 1999. *Bioactive ceramics: the effect of surface reactivity on bone formation and bone cell function*. *Biomaterials*. [https://doi.org/10.1016/S0142-9612\(99\)00181-7](https://doi.org/10.1016/S0142-9612(99)00181-7).

bone. In contrast, control implants of other materials slipped easily out of the bone because of the scar tissue formed at their interface (Fig. 7.13).

Several BGs were derived from the first Bioglass 45S5 composition by varying the percentage of SiO_2 , CaO , Na_2O , and P_2O_5 . Their ability to form bone bonding with time was evaluated in vivo. This thorough study described the relationship between glass composition, their dissolution/precipitation properties, and their bone-bonding ability. Along with the development of new biological assays, it has also been found that the dissolution products

of BGs stimulated osteogenic differentiation. Although these bioceramics are relatively “old” biomaterials, their ability to trigger bone formation is incomparable with other biomaterials. Nowadays, they benefit from new technologies allowing, on the one hand, to investigate the reason behind their bone-bonding ability, and on the other hand, to process them into high-performance scaffolds for bone tissue engineering.

Ducheyne, P., et al., 1994. Effect of bioactive glass templates on osteoblast proliferation and in vitro synthesis of bone-like tissue. *J. Cell. Biochem.* 56(2), 162–167.

Classical experiment

Degradation Behavior of Poly-DL-lactic Acid

Amorphous, noncrystallizable copolymers based on L-lactide, D-lactide, and glycolide show a surface to center differentiation upon hydrolysis, resulting in a hollowing out of the specimens during degradation.^{51,88} This phenomenon is related to the autocatalytic hydrolysis of the ester bonds in the main chain.

Until the publications of Vert and coworkers, the extent of degradation of these polymers both in vitro and in vivo was investigated by visual observation, viscometry, and changes in specimen mass. Polymer degradation was considered to be a homogeneous process, where water absorption throughout the polymer is followed by hydrolysis reactions. However, careful analysis of the molecular weight of the degrading polymer in 2 mm thick specimens showed a bimodal molecular weight distribution. It was found that in such poly(lactide)

specimens, the inner part of the specimen degrades at a higher rate than the outside.^{51,89}

When the specimen is placed in the aqueous medium, water penetrates into the material. Hydrolytic cleavage of the polymer chains occurs, and as carboxylic acid groups are generated, autocatalysis occurs as well. Within the specimen, degradation of the still insoluble polymer chains proceeds homogeneously via an autocatalytic mechanism. As soon as the molecular weight of the oligomers formed becomes low enough to allow solubility in the surrounding aqueous medium, these oligomers diffuse to the surface of the specimen and into the surrounding medium as they continue to degrade. This process, which combines hydrolytic degradation, diffusion, and solubilization, results in a differentiation between the rates of degradation at the surface and at the interior of the polymer specimen. As a result, hollow specimens can be formed during degradation, as shown in Fig. 7.14.

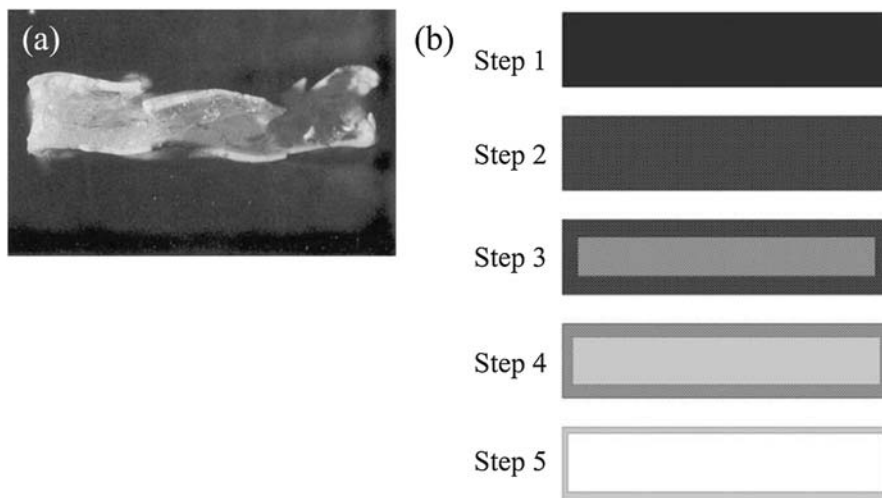


FIGURE 7.14

(a) Cross section of a PDLLA specimen degraded for 5 weeks in saline buffer. (b) Schematic representation of the different steps of the degradation of PDLLA specimens in aqueous medium. Step 1: initial specimen; Step 2: water absorption, start of ester bond cleavage, and decrease in molecular weight; Step 3: differentiation between surface and center, with dramatic decrease in molecular weight in inner part of the specimen; Step 4: diffusion of oligomers through thinning surface when molecular weight is low enough to allow solubilization in the medium; Step 5: hollow shell remaining after release of oligomers and slow degradation of the shell. *From Structure-property relationships in the case of the degradation of massive poly (α -hydroxy acids) in aqueous media. Li, S., Garreau, H., Vert, M., 1990. Structure-property relationships in the case of the degradation of massive poly (α -hydroxy acids) in aqueous media. Mater. Sci. Mater. Med. 1(4), 198–206.*

State-of-the-art experiment

Long-term behavior of degradable versus nondegradable bone scaffolds

In vivo animal experimentation has a key role in the evaluation of degradable scaffolds, with many factors being present in living tissue that cannot be easily simulated in vitro. Due to ethical, financial, and practical constraints, animal experiments need to be kept to the minimum required to answer a research question. Decisions need to be made regarding number of repeats, duration of experiments, and inclusion of appropriate comparators/controls. In many cases the ideal comparator is an empty defect as this will gauge if there is benefit of implanting a scaffold at all, rather than simply leaving the defect empty and establishing if it will self-repair. If the research question relates to whether the degradation itself will influence the tissue regeneration process, then it makes sense to compare a degradable scaffold to a nondegradable scaffold of similar architecture. This was the approach used by Ref. 90, where they compared a “fast” degrading PLGA scaffold to a “slow” degrading PCL scaffold and a nondegradable polyamide 66 (PA66) scaffold, for up to 12 months in vivo, in a rabbit model. All the scaffolds contained nano-hydroxyapatite (n-HA), as filler that is often included in bone scaffolds formulations, although with somewhat limited evidence of efficacy. Note that the comparators included in this

experiment will not tell us if n-HA definitively plays a role in the scaffold performance because comparator scaffolds of polymer (PLGA, PCL, and PA66) with no n-HA addition have not been included in the study.

So, what can be concluded from the study? Fig. 7.15a shows micro-CT scans of the three types of scaffolds at time points up to 12 months and alongside this (Fig. 7.15b) software analysis of the reconstructed images showing the ratio of bone volume to total volume (BV/TV). These values give an indication of the amount of bone regeneration that has taken place within the scaffold at the four time points. What is interesting that the BV/TV values of the n-HA/PLGA scaffold at 6 and 12 months are lower than its value at 3 months. Furthermore, at 12 months the BV/TV value of the n-HA/PA66 scaffold surpassed that of n-HA/PLGA and n-HA/PCL. It was observed that the n-HA/PLGA scaffold lost structural integrity, due to degradation, beyond 3 months. It seemed that, by retaining its structural integrity for longer, the nondegradable scaffold types supported more bone growth at the final 12-month time point. The key message here is that rate of degradation in relation to bone regeneration is critical, and it is important to perform experiments up to such time point as the degradation process is no longer influencing tissue repair.

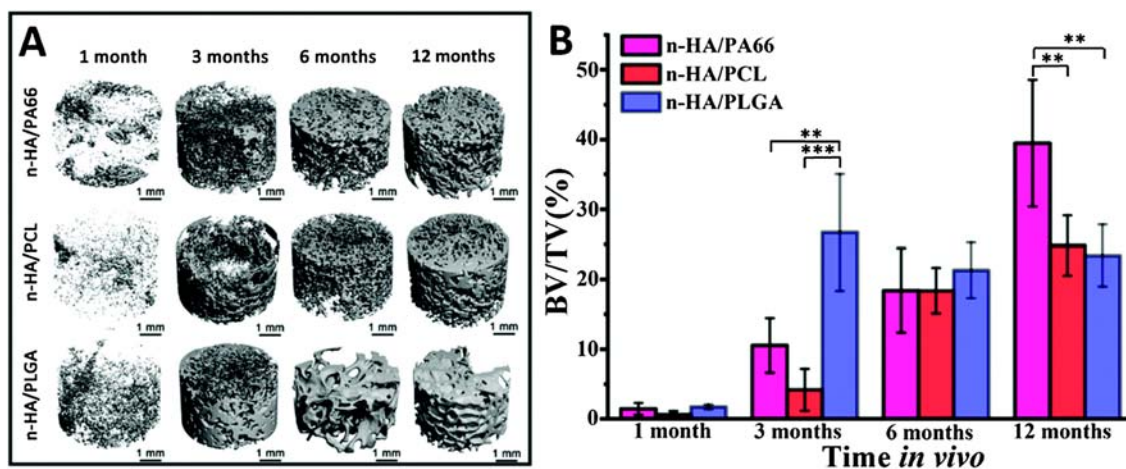


FIGURE 7.15

The 3D micro-CT reconstructed images of new bone tissue (a), and the quantitative BV/TV value (b) for the three scaffolds at 1, 3, 6, and 12 months after implantation. From *The long-term behaviors and differences in bone reconstruction of three polymer-based scaffolds with different degradability*, Huang, J., et al., 2019. *The long-term behaviors and differences in bone reconstruction of three polymer-based scaffolds with different degradability*. *J. Mater. Chem. B* 7, 7690–7703.

7.7 Recommended literature

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7.8 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter.
1. Define what is meant by the term biodegradable and how this differs from the term bioresorbable
 2. Summarize the main difference in mechanical properties between bioceramics, polymers, and biomaterials in terms of modulus, strength, and ductility.
 3. What is the key structural difference between a bioceramic and a bioglass?
 4. How can solubility isotherms be used to rank the dissolution rates of calcium phosphate bioceramics?
 5. List three factors in the design of a bioceramic scaffold that would influence its degradation rate.
 6. Beyond hydroxyapatite, what other calcium phosphate structures have found application in bioceramic scaffolds?
 7. What role might osteoclasts play in the degradation of bioceramic scaffolds?
 8. Describe the key distinguishing features of surface erosion compared to bulk erosion of polymers.
 9. For a hydrolytically degradable polymer, explain the role of initial molecular weight on degradation.
 10. With the aid of a sketch, describe the typical degradation sequence for a bioresorbable polymer such as poly-L-lactic acid (PLLA).
 11. Why does the local environment sometimes become acidic during degradation of a polymer?

12. Why might a polymer become brittle after melt-processing?
 13. How might the degradation rate of a polymer be accelerated to predict long-term behavior?
 14. What are the common methods of sterilizing biodegradable polymers and are any of these methods likely to have no influence on degradation behavior?
 15. Is degradation of polymers like to occur faster or slower in vivo compared to in vitro?
 16. What biomaterials have been explored of applications as tissue scaffolds?
 17. Why might alloys be used, as opposed to pure metals, for the development of tissue scaffolds?
 18. List three types of localized corrosion effects that are typically observed for biomaterials.
 19. Describe a method that can be used to establish the corrosion rate of a biomaterial.
 20. Give an example of a biodegradable composite and explain what this might be more challenging to process into a scaffold than a single-component biomaterial?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations.
1. What factors, relating to the in vivo environment, would influence degradation of biomaterials and which of these factors would particularly dominate the mechanism when considering the cases of bioceramics, polymers, and biomaterials?
 2. In a cell-free environment, bioceramics are known to degrade by a mechanism of physicochemical dissolution. Describe how the introduction of multinucleated cell plays a role in this process.
 3. In the context of polymer degradation, why might a patient experience inflammation and discomfort at the location of an implant?
 4. Give an example of a polymer that would gradually bioresorb in the body due to hydrolytic degradation. Describe this process referring to changes in molecular weight, strength, and mass during degradation.
 5. A bioresorbable bone scaffold made from PDLGA has an initial strength of 25 MPa and an initial molecular weight of 330,000 g/mol. To allow time for sufficient bone healing, the scaffold is required to retain at least 50% of its initial strength for 2 weeks following implantation. Establish whether the implant will achieve this.

$$k = 1.2 \times 10^{-6}/s$$

$$A = 8.5 \times 10^5 \text{ MPa/gmol}$$
 6. Outline the reasons why elevated temperature accelerated procedures need to be developed for bioresorbable polymers and the relevance of these in comparison to short-term biocompatibility testing.
 7. Review commercial medical device sterilization processes, including ethylene oxide (EO) and gamma irradiation. Outline how these processes may result in changes to the degradation performance of either bioceramic, polymer, or biomaterial scaffolds, being particularly conscious that EO sterilization involved a preconditioning step at high humidity.
 8. Outline the corrosion process of pure magnesium, describing how the specific degradation products might influence the in vivo environment.
 9. Describe how modifying the microstructure of a magnesium alloy can be used to control its degradation rate.
 10. Suggest how bioceramics, polymers, and biomaterials might be combined in the design of a hybrid, composite biomaterial, and what merits there might be developing such a material?

Challenge-based learning

Temporal control of scaffold degradation for allogenic islet transplantation

Note for teachers: A CBL user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Type-I diabetes (T-ID) is an autoimmune disease caused by the selective destruction of pancreatic insulin-producing beta cells, which results in uncontrolled hyperglycemia and hypoinsulinemia. Worldwide, one in every 500 people under the age of 19 suffers from type-I diabetes. Insulin therapy helps to manage the disease by keeping the insulin levels in acceptable range, but it is not a long-lasting cure. Therefore, there is a need to generate tissue-engineered beta cells and functional islets of Langerhans and to make them clinically available to treat T-ID. The long-term goal of this research field is to use allogeneic islets of Langerhans that can respond to postprandial glycemic levels and replace the function of the pancreas.

Motivation and stakeholders

Allogeneic islets can be transplanted in ectopic sites of the diabetic patient's body. To protect the transplanted cells from the host immune system, the islets are encapsulated in inert biomaterials. Still, allogeneic islands have a limited life span, and it is desirable that nonfunctional islands are cleared by the body. The same is true for the materials that encapsulate them. Solutions to address this problem should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as patients with T-ID, internal medicine doctors, cell biologists, biofabrication specialists, and biomaterial engineers.

Problem definition

Allogeneic island transplantation is a promising cell-based therapy for T-ID. However, allogeneic rejection is preventing this strategy to be widely used in the clinic. To overcome allogeneic rejection, a novel biodegradable scaffold, which protects the islets from the immune system, should be developed. The biodegradable scaffold should degrade

when the islet cells are not functional anymore. To do so, an inducible degradation-based switch (biosensor) should be developed. In summary, the islet cells should be maintained functionally during their life span and an inducible biosensor system should initiate the degradation to clear the remaining scaffold before implanting the new one.

Challenge

To design a noncytotoxic (biocompatible), polymer scaffold to be used safely and effectively to T-ID for allogeneic islet transplants.

Learning framework

Reading the Degradation of Biomaterials and Synthetic Biomaterials chapters and related literature will help you to understand:

1. What is a hydrogel.
2. The basic concepts of biomaterials degradation
3. The state of the art of allogeneic island transplantation.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

4. Strategies used in the body to control extracellular matrix degradation.
5. Molecular processes that are different between functional and nonfunctional islets of Langerhans.
6. Biomaterial engineering strategies to manipulate the degradation properties of a hydrogel.
7. The biochemical and molecular building blocks to engineer the degradation properties of hydrogels.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

7.9 Glossary

Additives are substances added in small quantities to improve the quality of a product.

Autocatalysis is where the reaction product is also a catalyst for the reaction.

Bioactive is having a biological effect.

Bioceramics are materials especially developed for medical and dental implants that directly interact with surrounding tissue to support tissue growth. Hydroxyapatite, alumina, and zirconia are commonly used.

Biodegradation is the gradual breakdown of the material mediated by specific biological activity in vivo.

Bioglass is a series of silica-based glasses with a three-dimensional SiO_2 network modified by the incorporation of Na_2O , CaO , and P_2O_5 .

Bioresorbable is a compound or a device that is totally eliminated or bioassimilated through natural pathways.

Block copolymers are two or more homopolymer subunits (blocks) linked by covalent bonds.

Chain scission is a term used in polymer chemistry describing the degradation of a polymer's main chain.

Critical sized bone defects are bone defects larger than 2.5 cm that will not heal within a patient's lifetime.

Foreign body response is an inflammatory response elicited by any material that would not normally be found within the body.

Gibb's free energy is a quantity that is used to measure the maximum amount of work done in a thermodynamic system when the temperature and pressure are kept constant.

Glass transition temperature is the temperature at which polymer molecules show macromolecular mobility and transition from a rigid state to a flexible state.

Host response is lodged by an immunocompetent individual in response to the presence of an antigen.

Hydrolytic degradation is the breaking of water-labile bonds of a polymer by water.

Mechanical degradation is the breakdown of molecules under the influence of mechanical stress.

Near-zero order kinetics is a type of reaction in which the rate of polymer mass loss is constant and independent of the influence of degradation products. This is seen in surface-eroding polymers.

Osteoconduction is the property of a material to support bone tissue ingrowth.

Osteoinduction is the stimulation of osteoprogenitor cells to differentiate into osteoblasts.

Osteointegration is the direct structural and functional connection between living bone and the surface of an implant.

Physiochemical properties are the intrinsic physical and chemical properties of a substance.

Reactive oxygen species (ROS) are a highly reactive and unstable group of molecules derived from molecular oxygen.

Stoichiometric refers to the relationship between the quantities of reactants and products before, during, and following chemical reactions.

Stress shielding is the reduction in bone density when an implant of higher stiffness than bone supports physiological loads.

Terminal sterilization is a process of sterilizing a product in its final container to ensure its sterility until use.

Thermal degradation is when a polymer changes its properties under the influence of heat.

Thermodynamic solubility is the saturation solubility of a compound at the end of a dissolution process.

© Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering. Some of this definitions were freely obtained and paraphrased from Wikipedia and Google.

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Cell–material interactions

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8.1 Learning objectives

After reading this chapter you will be able to:

- Understand the importance of cell–material interactions for tissue engineering.
- To understand the processes involved in integrin-mediated adhesion.
- Comprehend the process of mechanotransduction.
- To recognize that physical material properties, such as chemistry, stiffness, and topography, have strong effects on cell behaviors.
- Appreciate how the cell–material interface can be designed to harness cell–material interaction for therapeutic uses.

Ever tried. Ever Failed. No matter. Try again. Fail again. Fail better.

Samuel Beckett.

Any knowledge that doesn't lead to new questions quickly dies out: it fails to maintain the temperature required for sustaining life.

Wisława Szymborska, Nobel Literature Prize Winner, 1996.

8.2 Introduction

This chapter will discuss how cells interact with and interpret information from their extracellular environments. Tissue engineering techniques have been used to mimic important physical and functional components of cellular microenvironments, such as matrix topography (architecture), chemistry (e.g., ligand availability, charge), and mechanics (stiffness and viscosity). Engineering of these physical cues has significant effects on cellular behavior, such as the differentiation of stem cells. Understanding cell–material interactions will allow for the development of novel tissue engineering strategies for therapeutic applications, and as tools for investigating important cellular processes.

8.2.1 Cell–material/cell–extracellular matrix interactions

In this section, we will discuss the molecular mechanisms through which cells sense the mechanical properties of their environment. At the cell–material or cell–extracellular matrix (ECM) interface, sensing of the mechanical environment is a critical first step toward cell decision-making; as such, **mechanobiology** of cell adhesions is crucial for tissue homeostasis, development, and the outcome of many diseases. The ability of cells to sense and respond to mechanical stimuli is termed **mechanotransduction**. Sensing of the mechanical environment enables cells to rapidly respond to physical tissue parameters, influencing decisions regarding the form, function, and fate of the cells.

Mechanotransduction involves sensing of external forces and the transmission of this information, triggering a specific intracellular signaling response (see Chapter 4). The cytoskeleton plays a critical role in this process by linking the essential cellular components, e.g., cytoskeleton and the nucleus, to integrins, which together comprise the force sensing apparatus. At the cell–material interface, **solid-state interactions** confer effects through physical distortion, chemical signaling, adhesion points, or a combination of these. Physical distortion results from force transmission between cell and material due to spatial proximity. For example, ECM-induced distortion of the cell membrane can cause activation of stress-sensitive ion channels triggering downstream biochemical signaling cascades that result in force-dependent changes in cell behaviors. Emerging examples of intense research activity are receptors traditionally considered to be important in excitable cells (e.g., neurons and cardiomyocytes) but that are now being considered in mechanotransduction. Such receptors include transient receptor potential cation channels such as TRPV1 (transient receptor potential cation channel subfamily V member 1) and the piezo channels (e.g., piezo 1). These cation channels (typically Ca^{2+}) effect the cytoskeleton when mechanically activated and provide mechanoresponsivity to adhesion and cytoskeleton mediated tension derived from actin contraction.¹

Interactions mediated through chemical means follow similar mechanisms as soluble chemical signaling; for example, growth hormone and cytokine ligand binding to receptors resulting in activation/repression of signaling cascades (see Chapter 4). In solid-state interactions, these chemical factors are held in place through interactions with insoluble structures, such as the cell cytoskeleton or ECM fibers. In fact, most growth factors *in vivo* are bound to ECM components or are part of membrane complexes. Adhesion points at the cell–material interface are physicochemical connections between specialized subcellular sites at the cell membrane and specific components of the material surface.

8.2.2 Integrins

First, to understand how cells interact with the material interface we must first consider **cellular adhesion**. Cells express various cell surface adhesion receptors, including integrins, syndecans, cadherins, and other cell–cell adhesion molecules. The integrin family is a major class of transmembrane receptors and is the key player in mechanotransduction. Integrins are transmembrane heterodimeric proteins, with one α and one noncovalently bound β subunit, that act as mechanotransducers—that is, they are able to translate extracellular mechanical stimulations into intracellular biochemical responses. The α - and β -subunits combine to form one of 24 known integrins in humans (see Chapter 4). Cellular distribution of different types of adhesions is dependent on both cell type and the composition and mechanical properties of the ECM.²

Three domains make up integrins: a large extracellular domain, a membrane-spanning region, and, typically, a short intracellular cytoplasmic domain.² The large extracellular domain of the integrin contains a ligand binding site that binds specific **motifs** present in ECM proteins (see Chapter 5), such as

fibronectin (FN), vitronectin, and collagen. Integrin–ligand binding introduces conformational changes that unmask their short cytoplasmic tails, and this promotes linkage to the actin cytoskeleton and major signaling or regulatory proteins in the cytoplasm. The formation at the plasma membrane of direct linkage from ECM–integrin–cytoskeleton and associated signaling/structural proteins is demonstrated in Fig. 8.1 and is termed the **integrin adhesion complex (IAC)**.

Each integrin shares a significant amount of sequence homology and **cross-reactivity** between the ligands they bind. The most validated of these interactions is the amino acid sequence arginine–glycine–aspartic acid (RGD), which was first derived from FN, but is also present in other proteins. When an integrin binds its ECM ligand, it establishes physical contact between the cell and the extracellular environment. Ligand binding leads to transmission of information in both directions across the plasma membrane, termed **bidirectional signaling**, integrating the intracellular and extracellular environments. **Outside-in signaling** occurs when an extracellular ligand binds to the extracellular domain of an integrin and initiates a cascade of intracellular signaling events. **Inside-out signaling** occurs when cytoplasmic events result in a conformational change of the integrin, thereby changing its affinity for extracellular ligands.

Due to their range of intracellular interactions, integrin activation can result in a number of downstream effects, one of which is **integrin clustering**, whereby other integrins are recruited to the initial attachment site. Integrin clustering may be followed by structural and signaling protein recruitment

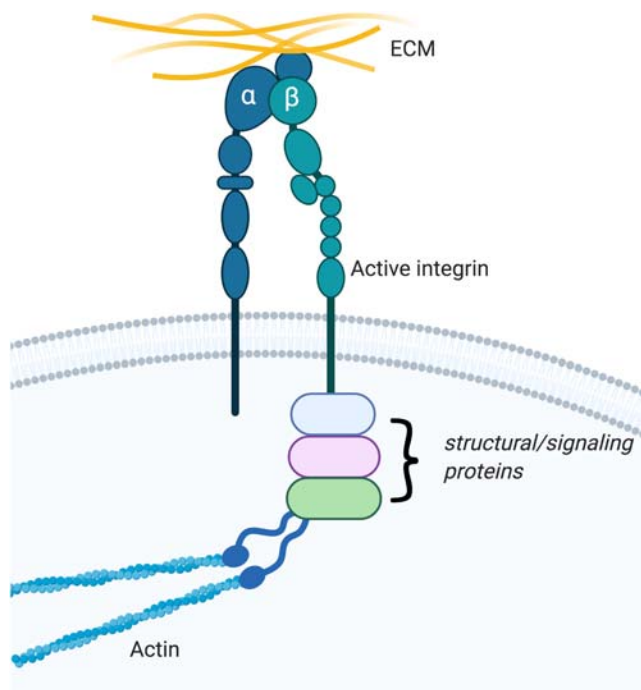


FIGURE 8.1

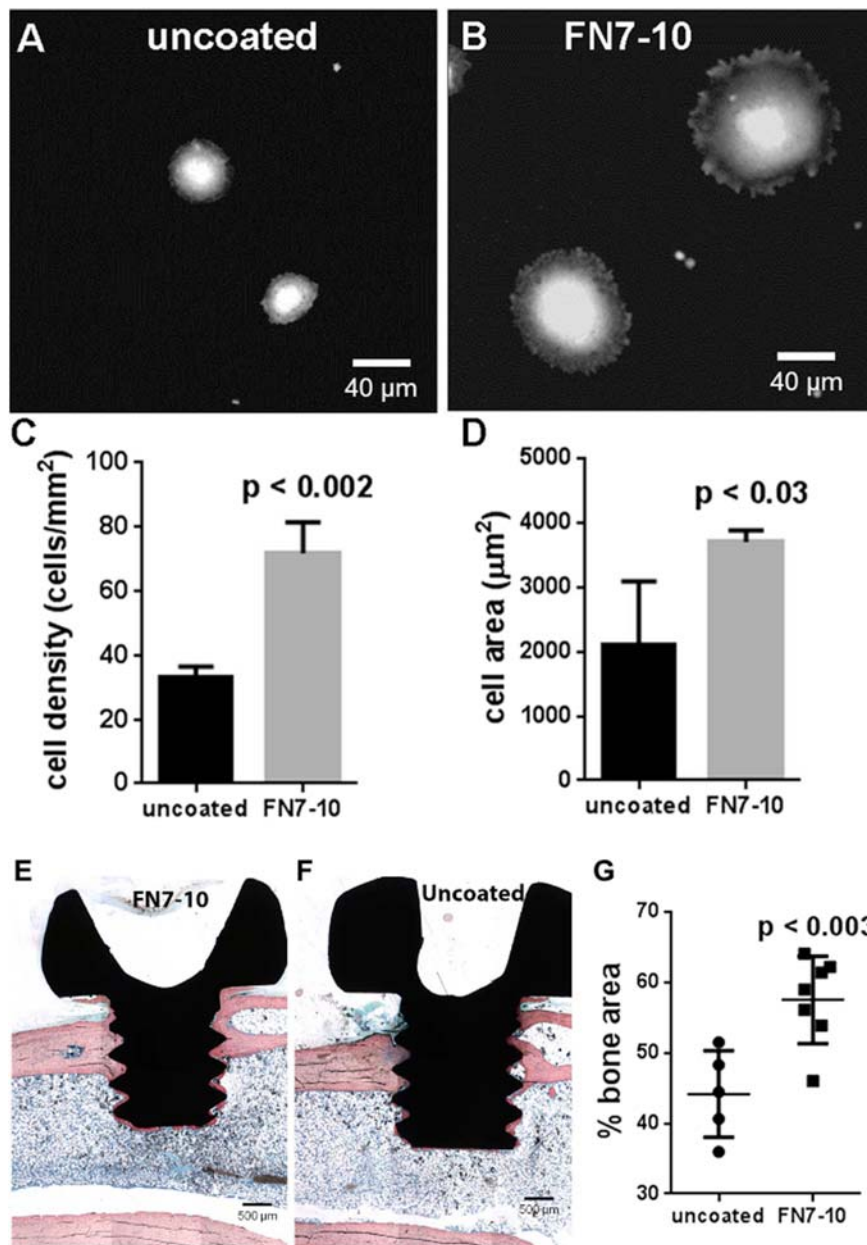
The integrin adhesion complex (IAC) at the plasma membrane, creating a direct link from ECM to intracellular components. *Created with BioRender.com.*

and connection with the cytoskeleton, leading to the formation of larger adhesion structures, changes in intracellular signaling, and thus cell behavior. Therefore, integrin binding to ligands is something tissue engineers can easily take advantage of by designing materials and scaffold systems that present, for example, the short peptide sequence RGD, to control cell behaviors, such as adhesion and differentiation, and improve scaffold outcomes upon implantation. For example, one study adsorbed FN7-10, a recombinant fragment of the abundant ECM protein FN containing the major integrin-binding RGD site, onto stainless steel screws used to fix bone implants (Fig. 8.2). In vitro they observed the coating promoted integrin-dependent adhesion and osteogenic differentiation of mesenchymal stromal cells (MSCs), and in vivo the screws significantly enhanced bone implant mechanical fixation.³

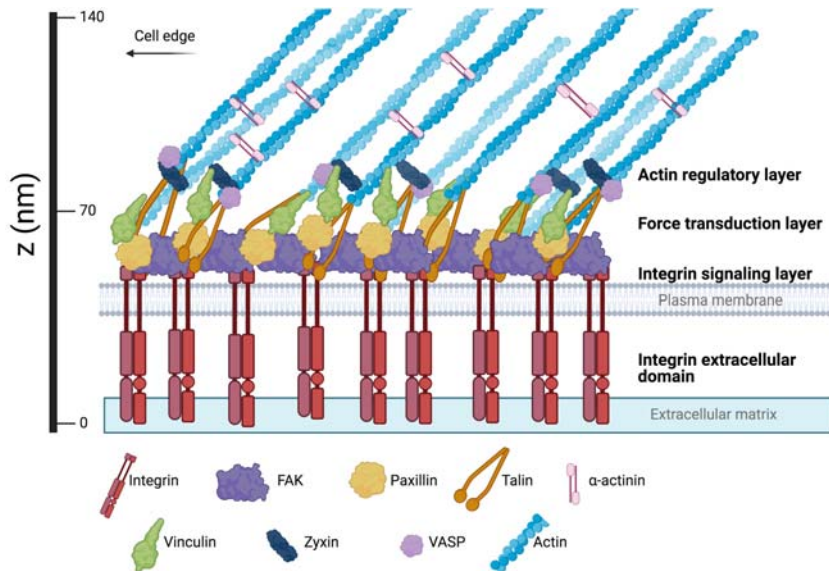
8.2.3 Integrin-mediated adhesion structures

The hierarchical structure of the linkage of the integrin actin is complex, with over 2000 proteins known to be associated in adhesion processes.⁴ The formation, disassembly, and maturation of IACs are tightly regulated in time and space. Cells probe the extracellular environment by forming membrane protrusions—lamellipodia or filopodia—that are driven by intracellular polymerization of actin filaments. Integrins in these protrusions bind ECM ligands leading to IAC formation, stabilizing the link between the actin cytoskeleton, and the ECM protein. Actomyosin contraction then generates **traction forces** on the substrate. Integrins can bind ECM ligands and form small IACs that are transient, with relatively short lifetimes. IACs that do not disassemble, instead enlarge and recruit more proteins to the adhesion site, these IACs then cluster to form **focal complexes**. Focal complexes further mature into larger, elongated supramolecular structures called **focal adhesions (FAs)**. Focal complexes may assemble without actomyosin-mediated force, and rapidly disassemble if no external force is applied. However, as soon as they connect to the force machinery (i.e., actin cytoskeleton), focal complexes mature into FAs.²

Adhesion formation and dynamics occur at the nanoscale, and as such, super resolution microscopy has been used to define the molecular architecture of FAs.⁵ Integrins and actin are separated by a ≈ 40 nm core region that consists of partially overlapping nano-organized molecular layers. The intracellular layer of FAs is comprised of scaffolding, docking, and signaling proteins that collectively serve as an interface between the transmembrane components directly linking the ECM to the actin cytoskeleton. Such FAs are large, dynamic macromolecular assemblies; in total, these plaques span <150 nm. Closest to the plasma membrane is the signaling layer, containing highly phosphorylated signaling proteins such as **focal adhesion kinase (FAK)** and paxillin. Next, the force transduction layer containing adaptor proteins, talin and vinculin, links integrins to the acto-myosin machinery. Talin proteins act as a tether, spanning the whole layer and regulating these ultrastructures—talin heads bind to integrins and talin rod domains directly bind to actin (up to 30 nm away from the integrins). These integrin–talin–actin complexes can then work as mechanical linkages, connecting the extracellular ECM to the intracellular cytoskeletal machinery. The most distal actin regulatory and actin stress fiber layers contain zyxin, VASP, and α -actinin, which act to stabilize actin filaments; these lie up to 60 nm away from the integrin layer. FA architecture and layer organization are demonstrated in Fig. 8.3.

**FIGURE 8.2**

Fibronectin fragment (FN7-10) coating of stainless-steel screws enhances cell adhesion (a–d) and osteogenic implant integration (e–g). From Agarwal R, et al. Simple coating with fibronectin fragment enhances stainless steel screw osseointegration in healthy and osteoporotic rats. *Biomaterials*. 2015;63:137–145.

**FIGURE 8.3**

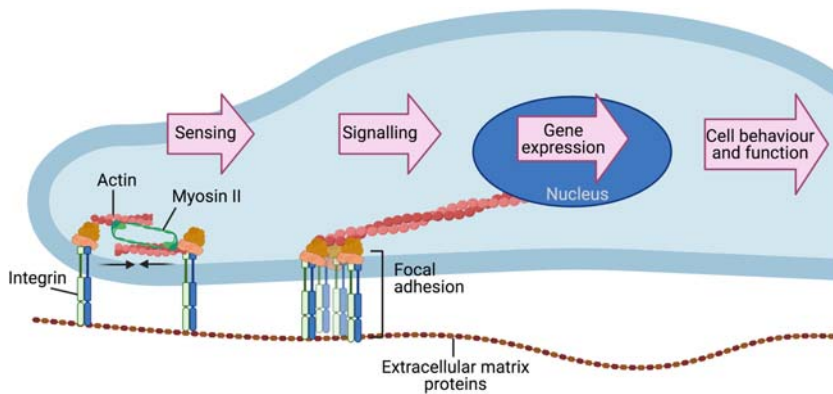
Focal adhesions contain hundreds of proteins that function to link the extracellular matrix to the actin cytoskeletal machinery. *Created with BioRender.com.*

8.2.4 Mechanotransduction

Upon ligand binding, intracellular integrin domains undergo conformational change, which through signaling protein activation (e.g., FAK) and/or physical propagation through structural cytoskeletal proteins (e.g., talin) leads to intracellular signaling. Therefore, integrins act as the mechanosensors and transmit information from the extracellular environment to produce downstream effects such as growth, apoptosis, differentiation, migration, and proliferation of cells.²

Many molecules are immobilized on the cytoskeleton. The cytoskeleton is thought to act as both a scaffold for solid-state biochemical reactions and as a reservoir for sequestered soluble factors, e.g., signaling molecules with cytoskeleton binding domains such as SH3 domains.⁴ We can say that if integrin signaling is sufficient to influence the molecular conformation of the cytoskeleton, then modifying the cytoskeleton shape, tension, structure, or kinetics will also influence any of the structures associated with the cytoskeleton.⁶ Where these structures are signaling molecules, and the sequestered molecules are released or binding sites exposed or hidden, downstream biochemical signaling cascades may be enhanced or inhibited following mechanisms similar to biochemical signaling cascades initiated by soluble chemicals, e.g., cytokines and growth factors. In this way, mechanical force can be transduced through the cytoskeleton and using solid-state chemistry can be converted into biochemical reactions (Fig. 8.4).

One of the first integrin signaling molecules to be identified was FAK. FAK acts as a phosphorylation–regulation signaling scaffold and is important for adhesion turnover, Rho-family GTPase-activation, cell migration, and cross-talk between growth factor signaling and integrins. FAK is ubiquitously expressed, and contains an N-terminal FERM domain, a central kinase domain, proline-rich regions, and a C-terminal focal adhesion-targeting domain that interacts with paxillin and talin. FAK represents

**FIGURE 8.4**

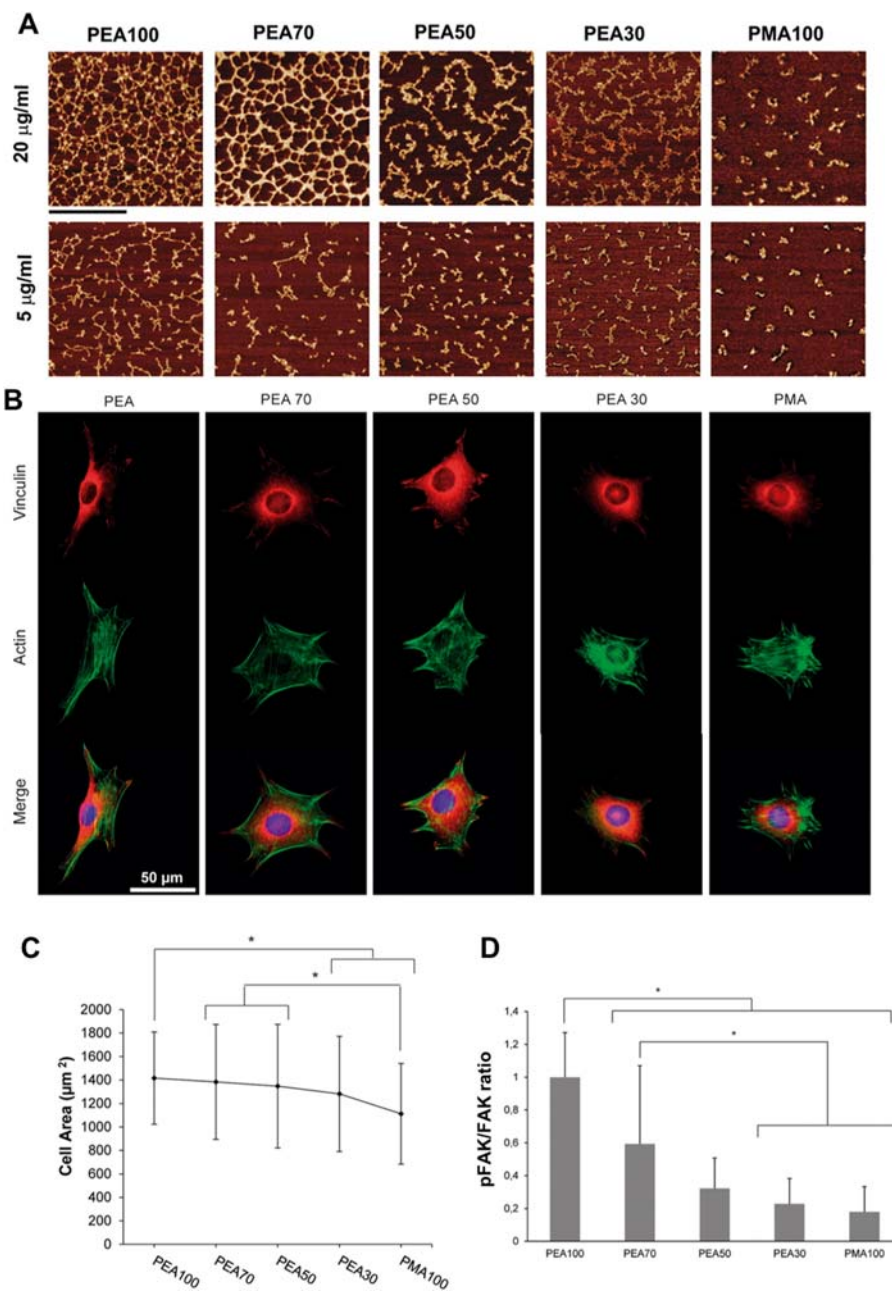
Mechanotransduction leads to changes in gene expression and cell behavior. *Created with BioRender.com.*

an example of outside-in signaling (i.e., from ECM to cell), in response to integrin-ECM binding leading to clustering, connection to the intracellular adhesion machinery and FA formation; FAK autophosphorylates and generates docking sites for SH2-domain-containing proteins, including Src kinases. These, in turn, become activated and phosphorylate FAK, promoting its kinase activity and its interaction with other proteins.

In addition to outside-in signaling, integrins can regulate their affinity for extracellular ligands. This occurs due to conformational changes in their extracellular domains in response to signals on the cytoplasmic tail; this is known as inside-out signaling (i.e., from cell to ECM). These signals cooperate with others to regulate cell behavior in a complex tissue microenvironment. For example, transforming growth factor- β (TGF- β) signaling can depend on integrin activation. TGF- β is produced in a latent complex with a latency-associated peptide tethering it to the ECM. Integrins are central to the mechanisms of releasing these key growth factors from the ECM, which they do by binding to the latency-associated peptide and coordinating matrix metalloproteases and other molecules at the cell surface to cleave the peptide and release the growth factor.²

8.2.5 Cell adherence to synthetic materials

The remainder of this chapter will discuss ways in which materials can be engineered to enhance cell interaction and gain control of phenotype and survival, and how this can be harnessed for clinical applications. However, it is important to note that cells do not tend to directly interact with synthetic material surfaces. Whether in vitro or in vivo, when cells encounter synthetic surfaces, a layer of proteins, from culture media or plasma, adheres to the surface within milliseconds. It is ultimately how protein adsorption, and thus ligand, e.g., RGD, presentation is affected by the properties of the material, such as surface charge, wettability (hydrophobicity), topography, and stiffness (see Chapter 6). It is this adsorbed layer of ECM protein, and thus the presentation of ligands, such as RGD sites, that is known to influence cell interactions with the underlying material.⁷ As such it is these cell-ECM-material interactions that the tissue engineer needs to understand and exploit when designing synthetic materials. Fig. 8.5 demonstrates how surface chemistry can be used to control adsorption of FN, and thus subsequent cell behaviors such as adhesion and

**FIGURE 8.5**

Surface chemistry controls vinculin and actin network assembly and cell adhesion, morphology, and signaling. From Mnatsakanyan, H. et al. Controlled assembly of fibronectin nanofibrils triggered by random copolymer chemistry. ACS Appl Mater Interfaces. 2015;7: 18125–18135.

spreading.⁸ Fig. 8.5a shows how different formulations of poly(ethyl acrylate) (PEA) and poly(methyl acrylate) (PMA) can affect the number of cells attaching to surfaces. Fig. 8.5b shows how the surface chemistry can affect the shape of cells. Cell shape is defined by proteins in the cytoskeleton like vinculin and actin. The size of cells on different surfaces can be quantified using image-processing methods (Fig. 8.5c). Additionally, surface chemistry can modify intercellular signaling pathways. When an important signaling protein like focal adhesion kinase (FAK) is activated via phosphorylation (pFAK) (higher pFAK/FAK ratio), then the cell can proliferate at a higher rate (Fig. 8.5d).

8.3 Surface chemistry

Surface chemistry refers to the chemical properties of the material surface in addition to any other modifications that are added to the surface; both of these aspects modulate cellular responses. As previously discussed, substrate chemistry affects the adsorption and protein presentation from the culture media to cells. For example, polystyrene, which is frequently used in cell culture, does not support cell attachment unless oxygen is incorporated, which in turn influences the deposition of FN.⁷ The chemistry of the material is not only relevant to how cells behave in the culture dish, i.e., through integrin interaction, yet is also important for clinical applications. Some medical implants encounter difficulties due to foreign body responses from unwanted blood–material interactions and inflammation in response to the material. Considering that proteins consist of hydrophilic/hydrophobic domains, charged groups, and polar/apolar regions, it is not surprising that engineering strategies focus on finding appropriate chemical compositions to influence these properties for controlling protein adsorption.

Here, we highlight essential key chemical features that determine protein adsorption characteristics, such as hydrophobicity and the presentation of chemical groups. Furthermore, we discuss how harnessing these concepts allows the creation of advanced platforms such as surface patterning and dynamic surfaces for studying mechanobiological concepts.

8.3.1 Hydrophobicity and hydrophilicity

Surfaces can be **hydrophobic** (“water-hating”), which repel water on the surface, or **hydrophilic** (“water-loving”). A bead of water on a hydrophobic surface will become rounded, a feature that can be measured using a technique called contact angle goniometry, which assesses the angle between the surface and the droplet of water. Hydrophobic surfaces have high **water contact angles** (>90 degrees), while a water droplet on a hydrophilic surface will have a low contact angle (<90 degrees) because the droplet can spread more on this surface. Extremely hydrophobic surfaces (known as superhydrophobic surfaces) can have contact angles >150 degrees.

Hydrophobic surfaces are not generally conducive to cell culture. The chemistry of some surfaces can be modified using plasma treatment, where a flow of oxygen plasma is used to introduce polar groups onto the surface to increase its hydrophilicity.⁹ This can be a temporary effect as some materials, such as the polymer poly(dimethylsiloxane) (PDMS), can recover their hydrophobicity with time. Typically, increasing the hydrophilicity of a surface promotes protein adsorption and attachment of cells, a result of, for example, integrin ligation and initiation of signaling cascades. As such, the degree of hydrophilicity can directly influence cell behavior, such as proliferation and differentiation. On the other

hand, hydrophobic surfaces can support the formation of organoids or embryonic bodies, as low cell attachment to the culture surface forces the cells to form aggregates.

8.3.2 Presentation of chemical groups

Next to hydrophobicity, the incorporation of functional groups also impacts protein adsorption and cell responses. These functional groups can include methyl ($-\text{CH}_3$), hydroxyl ($-\text{OH}$), carboxyl ($-\text{COOH}$), and amino ($-\text{NH}_2$) groups. For example, functionalizing glass surfaces with $-\text{NH}_2$ groups can promote osteogenic differentiation in MSCs, while $-\text{COOH}$ promotes chondrogenic differentiation.¹⁰ These effects are attributed to the polar charge of $-\text{COOH}$ and $-\text{NH}_2$ that increased protein adsorption. Because there exists a great variety of polymer compositions, high-throughput approaches are frequently applied to elucidate the best chemistry for a particular application (see Chapter 9). When such an approach is combined with analytical methods that describe functionalities of polymers in detail, additional correlations with cellular responses can be identified.

Functionalizing surfaces with chemical groups are also of interest for **medical devices** to prohibit a foreign body response and the accumulation of pathogenic organisms. A popular strategy is the grafting of polyethylene glycol (PEG) to surfaces (also known as PEGylation) to develop linear PEG brushes that resist the adsorption of numerous protein molecules. PEG derivatives and alternatives are continuously being developed to extend the persistence time of the surface chemistry in order to prolong the lifetime of the medical device.

8.3.3 Patterning using surface chemistry

Further to applying a single chemistry in the culture dish, it is also possible to pattern multiple different chemistries in one microenvironment. This allows directing where and how cells attach to the surface, leading to interesting platforms to research mechanobiology. PEG is often used to block adhesion since PEG discourages protein adsorption and thus precludes cell attachment. For cell research, combinations of PEGylated regions and hydrophilic regions coated with adhesive proteins allow the generation of “adhesive islands” where cell attachment can occur. The benefit of this approach is that the area of such an island is modifiable, allowing study of the effect of altering morphology on cell behavior. A first such study demonstrated a relationship between cell size and proliferation rate, with small cells on restricted adhesive islands proliferating less compared to unpatterned surfaces. Further to this, it has been demonstrated that apoptosis is initiated when cells are confined to very small adhesive islands, as the cells are unable to spread sufficiently and therefore do not activate the appropriate integrin-mediated survival signals.¹¹

Defined regions of surface chemistry can also be used to direct stem cell differentiation. This was illustrated by the confinement of MSCs to small adhesive squares, which predisposed MSCs to undergo adipogenesis, while larger adhesive squares stimulated osteogenesis.¹² This has been explained through changes in cytoskeletal tension and Rho/ROCK signaling. This study was one of the first studies demonstrating the relevance of mechanobiology for controlling cell behavior. Follow-up studies confirmed the observation that cytoskeletal tension strongly influences lineage specification. For example, when applying two different shapes (flower and star), but keeping the area constant, the star shape features induced greater cytoskeletal tension due to the generation of large actin stress fibers (Fig. 8.6). In contrast, on the more rounded features of the flower, cytoskeletal tension was reduced. The consequence for MSCs was that the star shape favored osteogenesis, while the flower shape induced adipogenesis.¹³

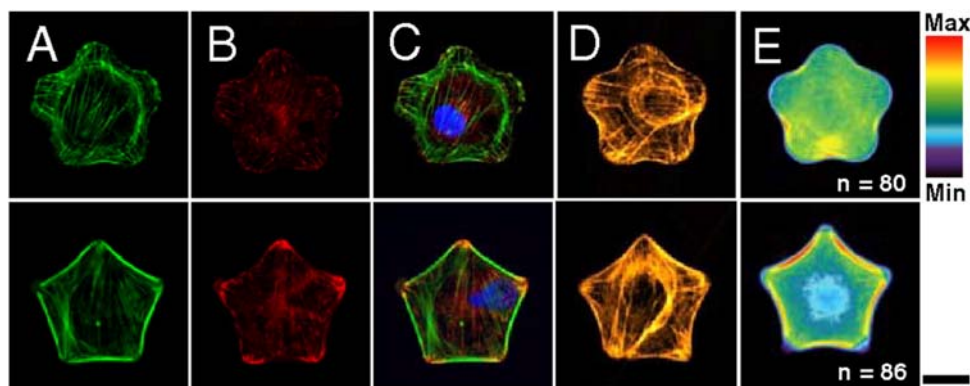


FIGURE 8.6

Geometric cues direct differentiation of mesenchymal stem cells. (a–d) Immunofluorescent images of F-actin (green), vinculin (red), and nuclei (blue), myosin IIa (yellow). (e) Fluorescent heatmaps of myosin IIa contractility. *From Kilian KA, Bugarija B, Lahn BT, Mrksich, M. Geometric cues for directing the differentiation of mesenchymal stem cells. Proc Natl Acad Sci USA. 2010;107:4872–4877.*

These examples demonstrate that adhesive features offer an excellent platform to investigate cellular architecture and physiology in a tightly controlled manner that can guide tissue engineers in, for example, design of medical implants.

8.3.4 Ligand spacing

As mentioned in [Section 8.1.2](#), integrin-mediated signaling requires the correct spacing and positioning of ligands from matrix proteins. This can be studied in detail with tightly controlled spacing and arrangements of surface chemistries, such as **self-assembled monolayers** (SAMs, e.g., self-assembling arrays of gold nanoparticles). For example, using gold nanoparticle SAMs conjugated to adhesive RGD ligands, it has been shown that the maximum interfeature distance capable of inducing integrin clustering in several cell types was 70 nm.¹⁴

Applying such techniques allows control of cell behavior. Similar to the case for adhesive islands, the spacing of the RGD ligand enables control of cell shape and cytoskeletal organization in MSCs, and subsequent lineage differentiation.² In endothelial cells, **ligand spacing** further influences sensitivity to growth factor signaling. When RGD-ligand spacing mimicking that of FN (44 nm) was presented, integrin activation and vascular endothelial growth factor signaling was increased, directly affecting endothelial cell migration.¹⁵ Other peptides can be applied to evoke distinct cell behavior, for example, introducing cell surface receptors that lead to T-cell activation (CD3) and natural killer cells (CD16), and influence immune cell responsiveness, which similarly was dependent on ligand spacing.¹⁶ It demonstrates that immune receptor triggering can be influenced by the nanoscale organization of receptor-ligand interactions.

8.3.5 Dynamic chemistry

Surface chemistries capable of being triggered to convert into different states in response to stimuli can be considered **dynamic chemistry**. These triggers can include changes in light, pH, temperature

stimulation, and changing electrical potential.¹⁷ Although most surfaces are designed to change states in an irreversible manner, a number of surfaces have been engineered to have a reversible switch. These platforms offer an intriguing research platform to study the mechanosignals required to switch cell behavior and offer the potential to influence biomaterial properties with a level of “on-demand” control for clinical applications.

One such method offering reversible stimulus-responsive changes involving light is through the creation of a monolayer that contains both PEG and c(RGDfK)-azobenzenes, of which the latter RGD-containing unit allows integrin binding and adhesion. UV illumination switches the azobenzene

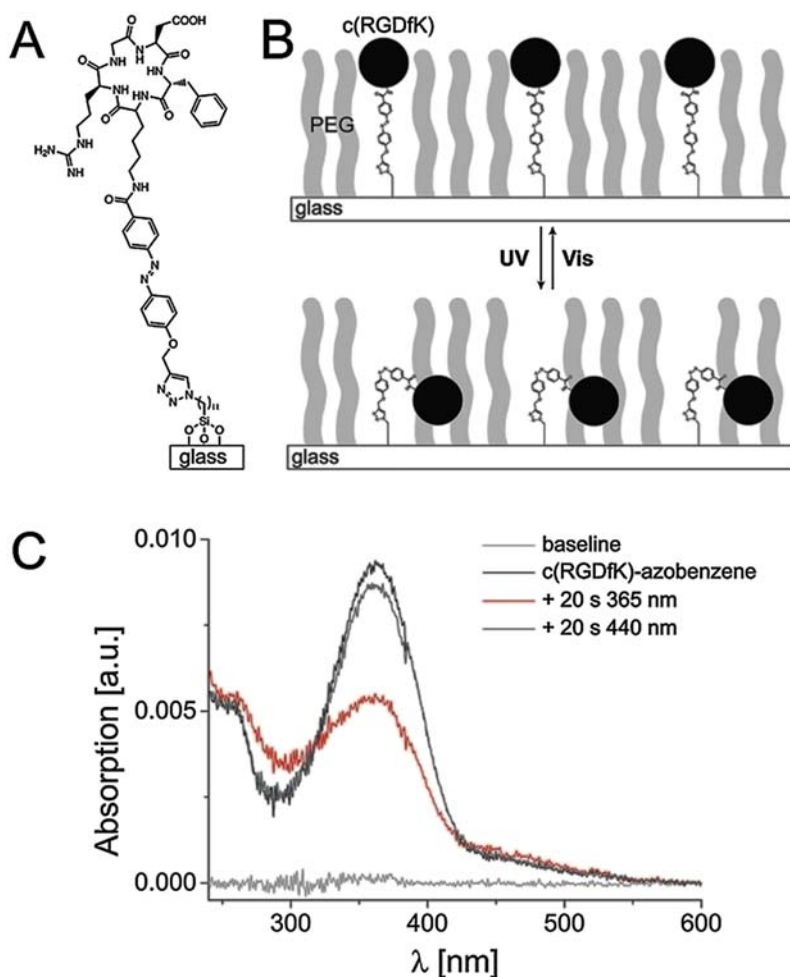


FIGURE 8.7

Reversible stimulus-responsive materials containing c(RGDfK)-azobenzene. (a,b) Structure of the c(RGDfK)-azobenzene, functionalized on PEG. (c) UV–vis spectra. *From Kadem LF, et al. Rapid reversible photoswitching of integrin-mediated adhesion at the single-cell level. Adv Mater. 2016;28:1799–1802.*

from the trans to the cis isomer, submerging the c(RGDfK) headgroup into the nonadhesive PEG background (Fig. 8.7). However, illumination of visible light allows rapid and reversible switching of the headgroup, Fig. 8.7c shows UV–vis spectra verifying reversible photoswitching of the mixed monolayer.¹⁸ Such an approach can also be achieved through “click” chemistry (modular reactions that generate larger molecules from smaller units), for example, where exposed or concealed adhesive RGD sites depend on the applied electrical potential. Altering the conformation of sulfonate (negatively charged) and ammonium (positively charged) terminated molecules in response to applied positive or negative electrical potentials facilitated the switch between exposure or concealment of adjacent RGD sites and thus allowed cells to migrate onto some areas of the pattern only when RGD was exposed.¹⁹

To conclude, engineered surface chemistries offer distinct functionalities for modulating cell–material interactions and thus cellular responses, including tunable surfaces to control processes such as cell attachment, signaling, migration, and cell fate.

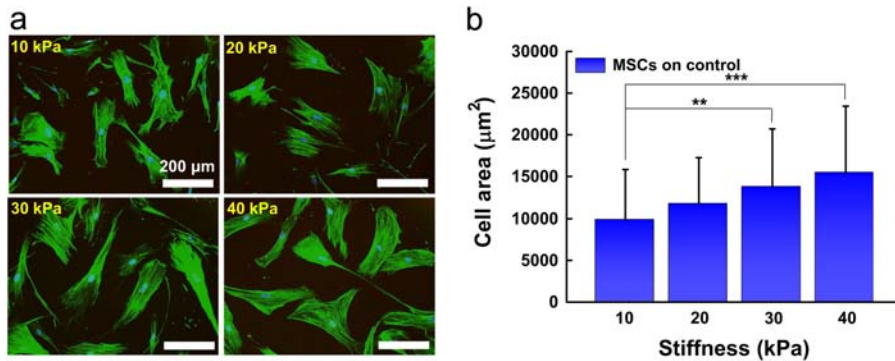
8.4 Material mechanics (stiffness)

Cells and their local microenvironment also communicate through mechanical cues, such as stiffness and viscosity, to regulate cell behavior, cell fate, and guide developmental processes. In embryonic development, mechanical forces are integral to organogenesis as the physical environment regulates cell behavior and ultimately function within a tissue. In adult tissues, adult stem cells require mechanical and physical cues from the ECM to retain their potency. In vitro, synthetic materials can be used to model the mechanical in vivo environment, providing platforms on which to accurately control and thus examine how cues such as matrix stiffness, applied forces, or viscosity, direct cellular behaviors. Such systems are fundamental to obtaining insights into the mechanobiology of processes such as development, stem cell differentiation and function, and thus to inform the design of tissue engineered scaffolds for regenerative therapies.

8.4.1 Cell behaviors and matrix mechanics

During embryonic development, cell-cell adhesions transmit tensile forces, and as development progresses, cells secrete ECM proteins to which they adhere, mechanically coupling them to the ECM in tissues by adhesion molecules such as integrins and FAs (see Chapter 3). Coupling of cell-ECM helps to drive morphogenesis and maintain the position and fate of cells in their microenvironment. Later, traction forces on this ECM regulate cell fate decisions during organogenesis, as cell layers are organized into defined structures, directing progenitor cells to diverse specialized functions in fetal organs. For example, **shear forces** from fluid flow of maternal blood promote fetal hematopoiesis and the morphogenesis of cardiac tissues.²⁰

This tissue remodeling and development continues into adulthood, as tissues maintain structure and function. Stem cells in the epidermal niche microenvironment, for example, use biomechanical signaling to regulate and balance proliferation and differentiation rates, and to coordinate cell fate with position in the niche. As the stem cells divide and cause local crowding, cell shape and stress distribution are mechanically deformed and trigger differentiation of the neighboring cell.²¹ Owing to the

**FIGURE 8.8**

Increasing substrate stiffness leads to increased cellular spreading in MSCs. (a) Immunofluorescence images, green = actin. (b) Quantification of cell area. Lee et al. *Controlling cell geometry on substrates of variable stiffness can tune the degree of osteogenesis in human mesenchymal stem cells*. *Mech Behav Biomed Mater*. 2014;38:209–218.

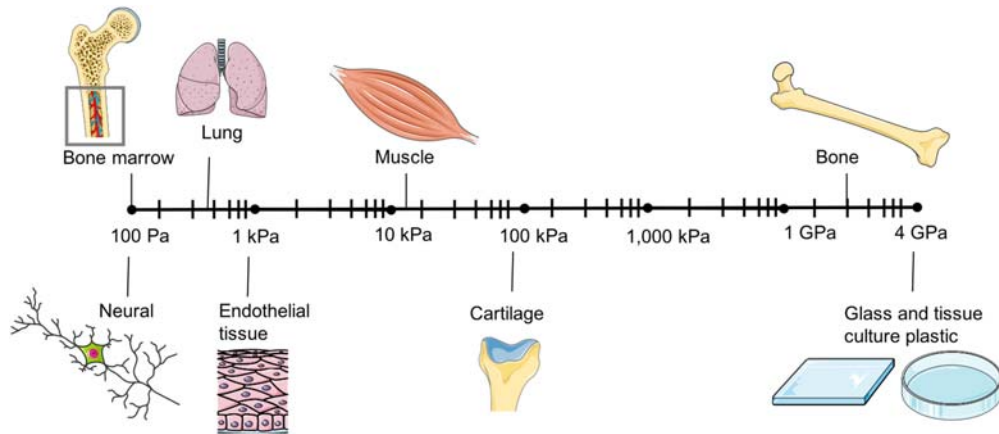
complexity of these developmental processes, research aiming to recapitulate them in vitro will require complex, highly controlled engineered systems.

These processes rely on the force sensing apparatus, FAs and the cytoskeleton, to sense substrate stiffness. Studies analyzing the effect of stiffness on various cell types in vitro have demonstrated and characterized cellular response by measuring cell spreading or area, cell migration, differentiation, proliferation, FA area, cytoskeletal polarization, and cellular traction forces. These studies highlight that cells are unable to spread on softer substrates, while spreading is enhanced on stiffer substrates, cells prefer to migrate from softer to stiffer regions, FAs are larger on stiffer substrates, the cytoskeleton is more tensile, and cells exert higher traction force on stiffer substrates. Fig. 8.8 shows human MSC spreading increases as substrate stiffness increases on PA gels of stiffness ranging from 10 to 40 kPa.²² Many cell types—MSCs, neural cells, myoblasts, fibroblasts, epithelial cells, and neutrophils—have been shown to have the ability to, and rely on, sensing substrate stiffness for survival. For example, in vitro cultures of neural cells are known to require soft substrates (between 100 and 500 Pa) to enhance cell survival,²³ whereas liver hepatocyte and stellate cells have a higher proliferation rate when cultured on stiff substrates, undergoing growth arrest or quiescence when cultured on softer substrates.²⁴

8.4.2 Mimicking tissue stiffness in vitro

Tissue engineering uses various techniques to create synthetic **microenvironments** that allow study of how mechanical cues regulate cells in vitro. This allows the ability to manipulate properties of the cell's microenvironment in a more predictable, defined, and controlled manner than in vivo modeling. Systems have been developed to allow study of how mechanical forces affect individual cells, or multicellular tissue models.

Tissue culture plastic (TCP), or polystyrene, is the classic cell culture model. However, TCP is a very rigid substrate, with a **Young's modulus** higher than that of bone (Fig. 8.9); it adsorbs serum proteins and

**FIGURE 8.9**

Biomechanical properties of various tissues in terms of stiffness (elastic modulus) in kilopascals (kPa). Created with [BioRender.com](#).

cell-secreted proteins—such as ECM proteins—in a nonspecific manner. Thus, it does not allow precise control over physical or biochemical cues that are presented to cells. Undefined basement membrane (Matrigel), collagen hydrogels, or decellularized tissues that yield native ECM are commonly used to provide a microenvironment more representative of native tissue stiffness to cells *in vitro*; however, these have poorly defined composition and vary batch-to-batch, making it difficult to discern effects from biochemical or physical cues. As such, synthetic polymers that mimic or incorporate recombinant ECM proteins have been developed, and these allow precise control over both mechanical and biochemical properties.

The need for high precision and reproducibility when fabricating substrates that vary in stiffness has led to the widespread use of synthetic polymers such as polyacrylamide (PA), PDMS, PEG, and polylactic acid (PLLA) whose resultant stiffnesses can be controlled by careful manipulation of the extent to which the polymer molecules are allowed to form cross-links with one another. This, in general practice, is achieved by subtle alterations to polymer concentrations or tuning of the pH and/or temperature during the cross-linking process. These polymers, however, lack **biocompatibility** due to a lack of cell adhesive ligands, so are often coated with ECM proteins, e.g., laminin, FN, collagen, or engineered to incorporate cell recognizable peptide sequences, e.g., RGD. For example, hyaluronic acid hydrogels engineered to present both cell–ECM (RGD) and cell–cell (HAVDI; adhesive domain from N-cadherin) ligands were engineered to precisely control presentation of each ligand. Modulation of ligand presentation was used to mimic mesenchymal developmental events, where cell–cell interaction dominates in early stages, progressing to increased cell–ECM interactions in later stages.²⁵ Another application demonstrated control over MSC phenotype, by engineering the gels in the physiological stiffness range of cartilage and presenting increased HAVDI ligands led to chondrogenesis.²⁶ This system lends itself well to cartilage tissue engineering, as chondrocytes typically live in pairs, and as such require increased cell–cell contact.

8.4.3 Stem cell differentiation and substrate mechanics

Interactions between stem cells and matrix stiffness may be a crucial factor in determining early-stage development, and in regulating adult stem cell behavior. ECM mechanics play a considerable role in guiding differentiation of embryonic stem cells during organogenesis, as well as providing cues to maintain their pluripotent state.²⁰ The lack of physiological-like cues provided by classic TCP systems leads to spontaneous differentiation of MSCs into clinically invaluable cell types, such as fibroblasts. Thus, synthetic tissue engineering strategies that recapitulate how mechanical forces regulate stem cells have been advanced, providing both insight into development, and the differentiation potential of stem cells to be harnessed for clinical use.

Fig. 8.10 demonstrates the range of stiffness of multiple tissues, and it has been shown that by mimicking the native stiffness of these tissues, stem cell differentiation can be guided toward the desired lineage.²⁷ This landmark study demonstrated that when MSCs were seeded onto PA gels with a range of stiffnesses, they developed branched, spindle, or polygonal morphologies when grown on substrates in the stiffness range brain (0.1–1 kPa), muscle (8–17 kPa), or stiff cross-linked-collagen substrates closer to the stiffness of bone (25–40 kPa). Further, the neuronal cytoskeletal marker $\beta 3$ tubulin is expressed in branches (arrows) of MSCs only on the soft matrices. The muscle transcription factor MyoD1 is upregulated and localized to the nucleus (arrow) on myogenic matrices. The osteoblast transcription factor CBF $\alpha 1$ (arrow) is expressed only on stiff substrates.

Cellular sensing of changes in ECM stiffness is mediated via the localization of receptor–ligand interactions, through FAs that generate intracellular cytoskeletal tensional states.² The extent of resistance or deformation of a substrate experienced by a cell results in increased or decreased **intracellular tension**

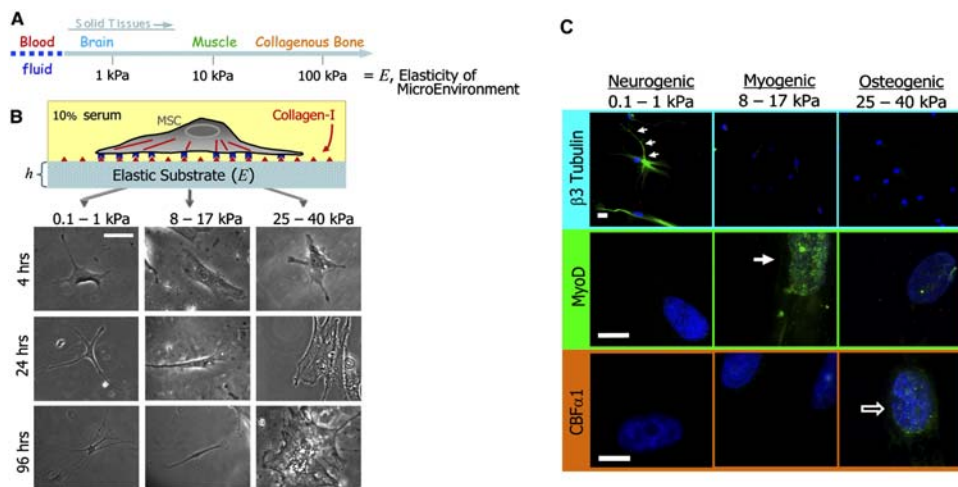


FIGURE 8.10

Matrix stiffness directs lineage specification of MSCs. (a) scale of stiffnesses (E) exhibited by solid tissues. (b) Light microscope images. (c) Immunofluorescence images of lineage specific markers. *From Engler A, et al. Matrix elasticity directs stem cell lineage specification. Cell, 2006;126(4):677–89.*

within it, facilitated by cytoskeletal contractility. This invariably has an effect on physical characteristics such as cell morphology, spreading, and adhesion strength. Loss of cytoskeletal tension on soft substrates, for example, leads to cells adopting a small, rounded morphology, while stiffer substrates, able to withstand higher traction forces by the cell, lead to a higher tensile state of the cytoskeleton and thus the cell is able to adopt a larger and more spread morphology (Fig. 8.10). These differences in physical characteristics brought on by cytoskeletal contractility, in stem cells, influence changes in broad signaling events culminating in changes in gene expression and cell differentiation.^{12,13} The formation of small, rounded stem cells on soft substrates leads to the expression of genes associated with differentiation into low tensile adipose cell types, whereas MSCs cultured on substrates that have a Young's modulus similar to that of nonmineralized bone (40 kPa) show an upregulation of Runt-related transcription factor (RUNX2) and early-stage signaling initiator BMPR-2 (bone morphogenetic protein receptor 2) which facilitate the formation of osteoblasts.²⁷

To further elucidate the mechanisms involved in mechanosensing, PA gels of varying stiffnesses have been used to investigate the events that lead to RUNX2 activation by the YAP/TAZ complex (Yes-associated protein/transcriptional coactivator with PDZ-binding motif) in MSCs. By varying substrate stiffness, it was found that if forces from adhesion and/or the cytoskeleton are high enough (above 5 kPa), the cytoskeleton is reinforced by stress fibers, mechanically coupling it to the nucleus. This provides a direct link from FA to the nucleus and leads to nuclear flattening, stretching of nuclear pores and thereby increasing YAP nuclear import. Fig. 8.11 demonstrates this is FA dependent, as when expression of FA-associated protein Talin 2 is knocked down using short hairpin RNA, cells cannot sense the underlying matrix stiffness. The nucleus is not connected to the force sensing machinery, and YAP remains cytosolic on stiff substrates.²⁸ As osteogenesis of MSCs occurs on stiff substrates (above 5 kPa), here

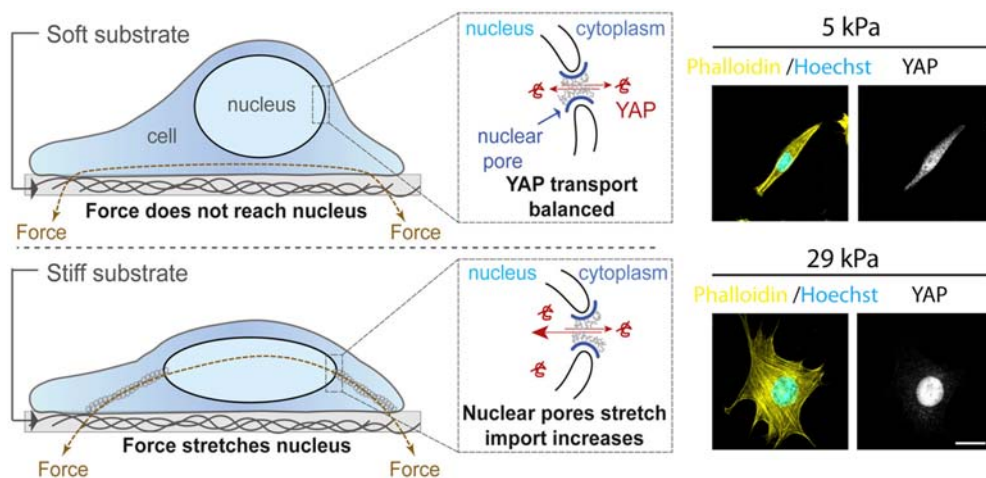


FIGURE 8.11

Mechanical coupling of extracellular matrix (ECM) to the nucleus leads to translocation of YAP from the cytoplasm to the nucleus on stiff substrates. From Eloague-Artola A, Andreu I, Beedle AE, et al. Force triggers YAP nuclear entry by regulating transport across nuclear pores. *Cell*. 2017;171 (6):1397–1410.e14. <https://doi.org/10.1016/j.cell.2017.10.008>.

YAP translocates to the nucleus via this mechanosensitive mechanism and consequently activates RUNX2, driving an osteogenic transcription program.²⁹

As discussed, control of material stiffness or mechanics can have profound effects on how cells sense and interact with their microenvironment, thus leading to widespread effects on cell behavior. Tissue engineering strategies that produce scaffolds with biomimetic mechanics have allowed insight into stem cell regulatory mechanisms and present a valuable method for production of clinically relevant implants.

8.5 Topography

Next, we shall consider physical patterning, or architecture, of the material surface—**topography**. Standard cell culture is carried out on flat, planar substrates. For cells, this is fundamentally different from the native 3D environment in which they reside, where physical structures provide extrinsic cues that will guide intrinsic cellular processes. Recapitulation of topographical features using tissue engineering techniques is a useful tool that provides a controlled and well-defined stimulus for influencing cell responses. Topographies can include micro- or nanostructures such as pits, pillars, and grooves, or physical properties such as roughness, Fig. 8.12 highlights some examples. Such physical cues have been implemented to investigate cell behaviors, such as stem cell differentiation, and to coat medical implants, such as hip replacements, to improve in vivo integration. In this section, we will discuss the range of effects topographies can have on cells.

8.5.1 Cell guidance by micro- and nanostructures

The fact that cells guide to topographical features has been known for over 100 years. The term **contact guidance** describes how cells align along topographical features, and was coined in the 1950s; then in 1964 Curtis and Varde showed that fibroblast cells can position themselves parallel to microstructures with diameters of 10–30 μm .³⁰ Microstructures are in the range of the size of the cell itself and thus lead to whole-cell responses such as cell alignment. In contrast, nanoscale features present physical cues that are several orders of magnitude smaller than that of the cell. At the nanoscale, as features are of a similar size to individual cell receptors, it is therefore possible, using surface design strategies, to target receptor-driven pathways such as adhesion formation, which will lead to control the cellular response (Fig. 8.13). So, contact guidance at the microscale is initiated as cells are confined and this will cause them to align their receptors through cytoskeletal remodeling. However, at the nanoscale, receptors will align and this will remodel the cytoskeleton and thus orientate the cell.³⁰ Cells are very sensitive to even very small changes in their nanoscale environment. Integrin containing filopodia at the cell leading edge initiates this receptor-driven contact guidance. Filopodial interaction has been seen with features as small as 8 nm in size. On the larger scale, nanoscale features have been shown to imprint their morphology into the cytoskeleton of adhered cells, with this pattern mimicking being broken by inhibition of adhesion.³⁰

Micron sized features, such as microgrooved substrates, are commonly employed in nerve research and microgrooved nerve “bandages” have been trialed in the clinic for peripheral nerve repair. For this application, the direction of axonal growth is important, and grooved structures have been

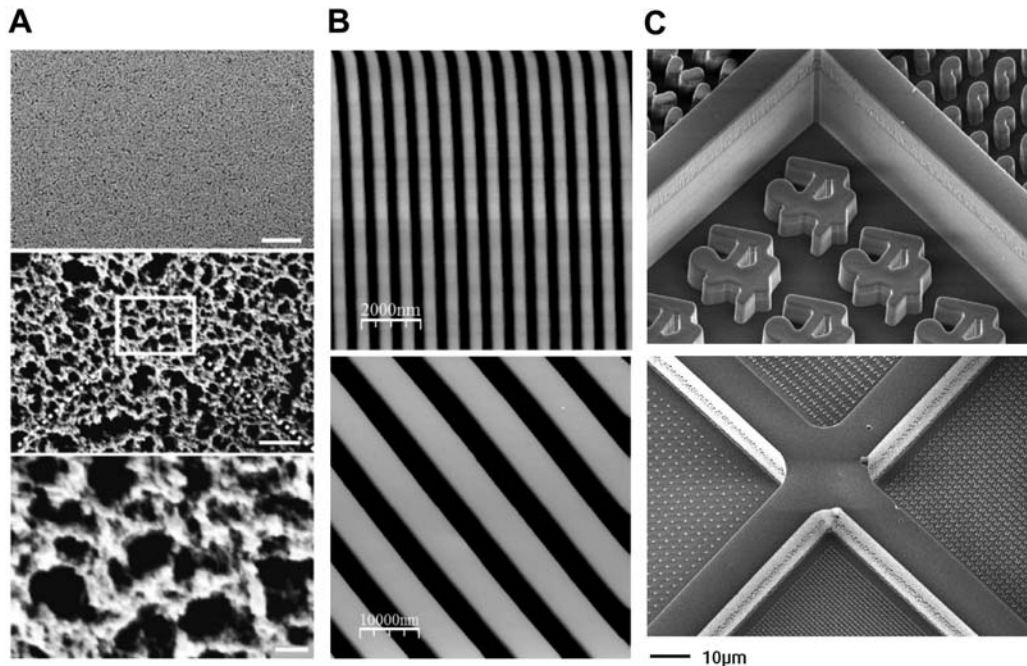


FIGURE 8.12

Scanning electron microscopy image examples of surface topographies. (a) Surface roughness. (b) Grooves. (c) High throughput topography platforms. (a) From Jaggy M, et al. Hierarchical micro-nano surface topography promotes long-term maintenance of undifferentiated mouse embryonic stem cells. *Nano Lett.* 2015;15: 7146–7154. (b) From Pan F, et al. Topographic effect on human induced pluripotent stem cells differentiation toward neuronal lineage. *Biomaterials.* 2013;34:8131–8139. (c) From Hulshof FFB, et al. NanoTopoChip: high-throughput nanotopographical cell instruction. *Acta Biomater.* 2017;62:188–198.

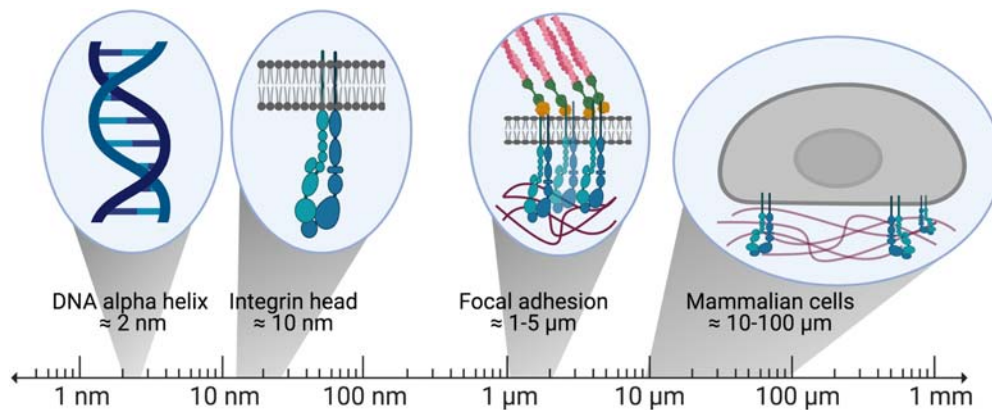


FIGURE 8.13

Scale of cellular components. Created with [BioRender.com](https://www.biorender.com).

demonstrated to lead to contact guidance with whole cells, such as Schwann cells or neuronal stem cells, undergoing morphological changes to orientate with such structures (Fig. 8.14).³¹ In a similar vein, nanofibers created using techniques such as electrospinning can be used to guide cell orientation. This has been implemented in tendon and ligament repair applications, where human tendon–derived stem cells cultured on aligned nanofibers have been shown to lead to increased expression of integrins, and nonmyosin II B required for integrin-mediated transport of collagen fibers and subsequent contraction.³²

Microscale pits have also been used to mimic osteoclast resorption, and to stimulate bone formation from osteoblasts. In bone homeostasis, remodeling is required during bone growth, osteoclasts resorb bone by using acid to form resorption pits. Bone forming osteoblasts perceive these as features that they need to repair through deposition of bone matrix. Hence, tissue engineering strategies to mimic such features can stimulate new bone deposition.³⁰ **Surface roughness** is also regarded as a topographical cue that is also typically associated with bone tissue engineering applications. As a surface feature, it is less precisely controlled than the above-mentioned techniques and leads to randomly distributed

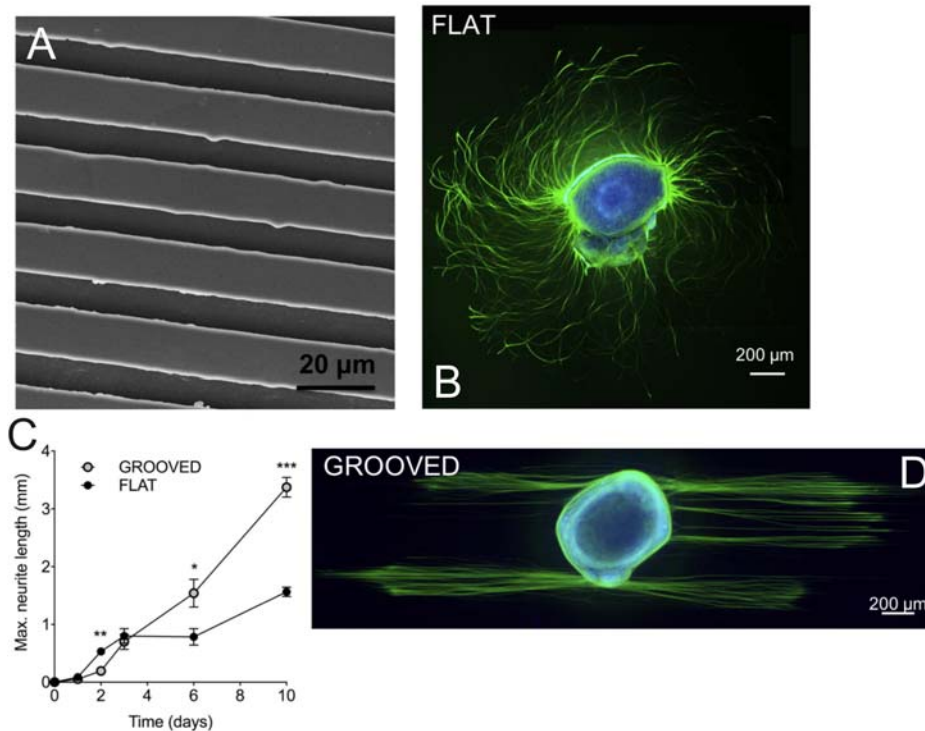


FIGURE 8.14

Dorsal root ganglia cultured on microgrooved substrates. (a) microgrooves. (b) quantification of neurite length. (c–d) green—b-tubulin. From Thomson et al. *Microtopographical cues promote peripheral nerve regeneration via transient mTORC2 activation*. *Acta Biomater.* 2017;60: 220–231.

features and both micro- and nanoscales. It has been demonstrated that increases in surface roughness can lead to osteogenic lineage commitment of MSCs and osteoblast proliferation, whereas the opposite effect is observed in fibroblasts. Techniques such as sand-blasting and acid-etching of titanium implants have been demonstrated to improve their osteo-integrative capacity.³³

8.5.2 Nanotopography and stem cell differentiation

First reports of cells responding to features with nanoscale dimensions came in the form nanogrooves organizing cell alignment in the 1990s, aided by development in lithographical techniques. The first reports of cell response to all nanoscale features came 10 years later through use of polymer phase separation to form features. This was followed by use of electron beam lithography, a technique to write smaller and smaller transistor patterns into silicon wafers for the electronics industry, which paved the way for study of precisely controlled patterns to better understand cell response.³⁰

MSCs have been extensively studied for topographical interaction effects. The involvement of nanotopography on MSCs differentiation down the osteogenic lineage has been closely studied for over a decade. Using techniques such as electron beam lithography to design cell culture platforms with precise features has enabled investigation into the link between adhesion and osteogenesis in this cell type. Nanopits of 120 nm diameter and 100 nm depth were arranged with either an ordered square spacing of 300 nm centre–centre, or in disordered arrangements with a centre–centre offset of ± 50 nm. On the disordered surfaces osteogenesis was observed—MSCs formed large, supermature FAs and intracellular tension is increased as a result. These mechanical changes led to increased signaling through pathways such as ERK1/2, which can subsequently phosphorylate and activate the master osteogenic regulating transcription factor RUNX2, while simultaneously inactivating the adipogenic factor peroxisome proliferator–activated receptor γ , thus stimulating osteogenic differentiation (Fig. 8.15).³⁴ These changes in intracellular tension can also lead to further changes in nuclear shape, consequently affecting

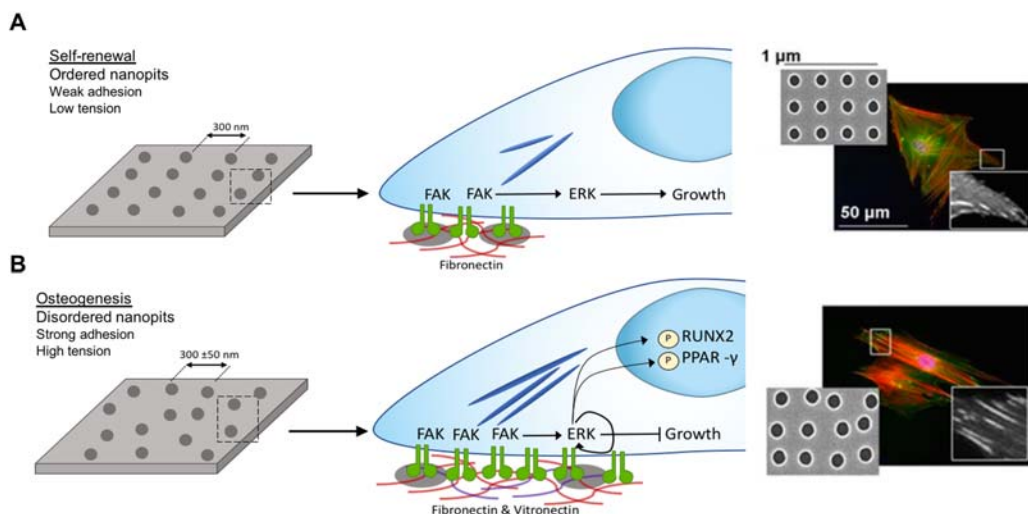


FIGURE 8.15

Nanopit topographies control MSCs self-renewal **(a)** and differentiation **(b)** through control of adhesion formation. *From Donnelly, Salmerón-Sánchez, Dalby. Designing stem cell niches for differentiation and self-renewal. J R Soc Interface. 2018.*

chromosomal arrangement, which can further impact gene expression and stem cell phenotype.³⁰ This technology has since been translated toward the clinic, by engineering titanium with disordered nanopits to improve success of orthopedic implants.³⁵

In contrast, on the ordered surface, adhesion and intracellular tension was reduced and the nanopit topography then supported self-renewal of MSCs.³⁶ This is important, as it shows that very slight reconfigurations of topography can have large effects. Control of both self-renewal and differentiation is important if we wish to grow large amounts of MSCs for cell therapies. MSCs are recognized for their therapeutic potential in regenerative medicine and tissue engineering where differentiated cells can be used to aid regeneration and also in, for example, transplant therapies where their immunomodulatory phenotype can help transplants engraft (e.g., islet transplantation). For these approaches, however, the MSCs must first be grown to large numbers so that “doses” of therapy can be manufactured; cell number expansion is required to make this sector cost-effective and to support emerging industry.²⁹ Topography has given us the ability to start to define the rules of maintaining self-renewal *ex vivo*.

MSCs have acted as pathfinder cells for understanding the potential of topography in stem cell control. This is because they are simple to culture and to grow on materials, not usually requiring complex coatings or feeder layers typical of embryonic stem cell (ES) culture. However, nanotopography strategies to mimic the *in vitro* microenvironment of ES cells have been investigated as a potential method to remove the need for feeder layers, and control ES pluripotency long term. One study investigated ES self-renewal using topographical surfaces with a range of nanoroughness. Here, smooth surfaces (1 nm) were shown to support stemness, whereas nanorough surfaces (70 and 150 nm) led to a loss of pluripotency.³⁷ Here, the molecular mechanisms of sensing substrate architecture are similar to those discussed for MSCs, where control of adhesion formation and thus intracellular tension leads to control of mechanosensitive signaling pathways that ultimately control stem cell fate.

To conclude, topography has broad effects on cell activity and is a powerful surface cue that can be used to investigate basic cellular mechanisms and processes in many cell types. Topography has also seen advancement toward the clinic, demonstrating that harnessing these cellular responses can be translated to improve tissue engineering strategies. High throughput screening platforms allow investigating multiple topographical cues in one system due to the geometrical complexity of topographical cues.³³ More recently, platforms have been developed to investigate the effects of multiple environmental cues in one system (see **State-of-the-art experiment**).

8.6 Future perspective

This chapter has discussed how cells interact with material platforms, and how the mechanosensitivity of cells can be investigated to elucidate regulatory mechanisms and harnessed to engineer clinically relevant tissue engineering strategies. We are currently seeing advancements in technology associated with molecular and cell biological techniques (e.g., superresolution microscopy), material design and fabrication (e.g., 3D printing, high throughput fabrication platforms), and advanced computational analysis (e.g., machine learning).

Such advancements enable tissue engineers to continue to design more precisely controlled medical devices that more closely mimic the properties of the native microenvironment. These approaches are

beginning to see translation toward the clinic, where, for example, nano topographically coated orthopedics implants could economically, and without the use of chemical modifiers or growth factors, improve osteointegration and implant success.³⁵

Technological advancements are also leading to the development of culture platforms that enable investigation into cellular processes in a more *in vivo*-like manner. This is particularly valuable when investigating elusive microenvironments such as the bone marrow. Human bone marrow is almost inaccessible for real-time research, yet is a dynamic and vital organ, central to blood system homeostasis. Advances in tissue engineering techniques have allowed development of platforms designed to mimic physical and functional aspects of this microenvironment, in order to investigate cellular mechanisms fundamental to its function. For example, one platform engineered a hydrogel microwell system to present bone marrow-associated signaling proteins to hematopoietic stem cells at single cell resolution to assess gene expression signatures associated with lineage commitment in this cell type.³⁸

Furthermore, as cell therapies become increasingly applied in the clinic, these techniques present strategies to precisely control cell–material interaction in order to control cell growth and phenotype. This leads to the ability to reliably produce therapeutically relevant cell yields, without the need of complex chemical formulations typically required for stem cell differentiation.³⁹ Osteogenic media, for example, contains a complex cocktail of ascorbic acid, glycerophosphate, and dexamethasone. However, dexamethasone is also a major component of adipogenic media, resulting in nonspecific action and off-target effects. Therefore, by engineering platforms that control material–ECM protein–cell receptor interactions, we can negate the need to use crude, nonspecific, techniques and harness cell mechanosensing for advanced therapeutics.

Summary

- Cells–material interactions are mediated through ECM protein adsorption on the substrate surface.
- Integrins are the major receptor involved in sensing the mechanical environment.
- Mechanotransduction is the conversion of mechanical cues from the extrinsic environment, into intrinsic cellular biochemical signaling, that ultimately affects cells behavior.
- Control of adhesion formation can have profound effects on cell behaviors.
- Modifications to surface chemistry, stiffness, and topography can be engineered to control cell–material interactions.
- These techniques have been employed to investigate important cellular processes, such as adhesion dynamics.
- These tools are particularly useful for understanding and controlling stem cell phenotype, both differentiation and maintenance of stemness.
- Such techniques can be applied to medical devices or implants to help improve cell–material interactions *in vivo*, and thus device/implant success.
- Control over stem cell phenotype using such techniques is also clinically relevant.
- Technological advances continue to advance our understanding of cell–material interactions, and how this can be exploited for tissue engineering strategies.

Classical experiment

Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment

McBeath et al. (2004) have demonstrated that the seeding density at which mesenchymal stem cells (MSCs) are plated influences their lineage commitment, with high and low densities stimulating adipogenic and osteogenic differentiation, respectively. In this chapter, the authors identified why this density-regulating lineage commitment happens and the mechanisms that control it, namely mechanical forces.

Using microcontact printing the authors fabricated an array of adhesive FN islands surrounded by a

nonadhesive substrate, which forced the cells cultured upon it to grow individually (one on each island). Cell shape and the degree to which these individual cells spread were defined by controlling the size of the islands. These experiments allowed the effects of cell shape to be isolated from other factors of the local tissue microenvironment (such as cell–cell contact inhibition). The authors reported that cell shape alone can regulate MSC lineage commitment, with flattened spread cells committing to an osteogenic lineage and unspread, round cells an adipogenic lineage (Fig. 8.16).

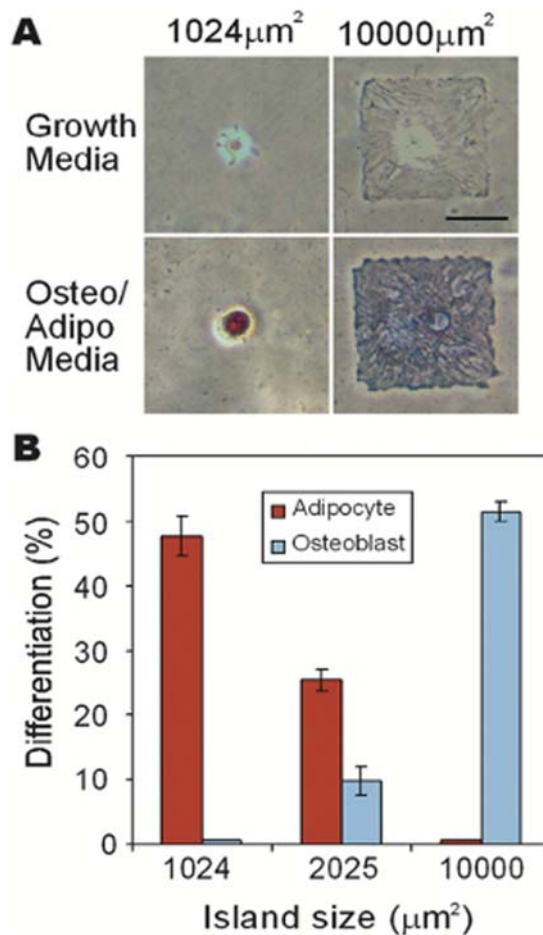


FIGURE 8.16

Cell shape—derived lineage commitment. (a) Light microscopy images, lipid stain = red; alkaline phosphatase stain = blue. (b) Percentage cell differentiation. From McBeath et al. *Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment*. Dev Cell. 2004;6:483–495.

Classical experiment—continued

The mechanisms regulating this shape-mediated signaling were found to involve the actin cytoskeleton and RhoA GTPase activity, with both an intact cytoskeleton and RhoA being central to the process—neither of them alone was sufficient to shift lineage commitment. RhoA is a central regulator of cell contractility in many cells and has a number of effectors, including some that influence cytoskeleton structure and function. RhoA activity was found to be greater in spread than unspread cells and thus corresponds with the authors' complementary observation that continually active RhoA stimulated osteogenic lineage commitment, while inactive RhoA decreased osteogenic commitment. Remarkably, RhoA activity was found to be a stronger stimulus than traditionally used chemically mediated differentiation

signals—demonstrating the power of cell shape to control stem cell differentiation. RhoA is known to play a central role in soluble factor signaling and is thus identified as a potential integrator of structural and soluble cues.

These experiments were fundamental to our understanding of the power by which mechanical forces, embodied by cell shape, cytoskeletal tension, and RhoA signaling, can control stem cell differentiation.

McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell. 2004;6:483–495.

State-of-the-art experiment

Discovery of synergistic material—topography combinations to achieve immunomodulatory osteoinductive biomaterials using a novel in vitro screening method: The ChemoTopoChip.

After reading this chapter, it will become clear that multiple material types influence the culture environment. Possibilities include topographical cues or changing the chemistry of the substrate. These applications are helpful for regenerative medicine by beneficially altering cell behavior. However, due to their enormous design space, it is challenging to identify the most optimal condition for a specific application, such as stem cell maintenance or differentiation. In the topographical design space, geometries exist in many different shapes and sizes, such as pillars, grooves, and pits, both in micro and nanodimensions. Similarly, the chemistry of the substrate can be quite diverse. For example, for polyurethane alone, an FDA-approved biomaterial, hundreds of different variations exist.³³

In an attempt to explore the individual effects of a topographical or chemical cue in this enormous design space, researchers developed high-throughput screening platforms. For topographies, platforms exist such as the TopoChip with 2176 unique geometries⁴⁰ or the BSSA platform

with 504 structures.⁴¹ For studying the chemical design space, combinations of monomers can be printed on a substrate leading to unique polymers. As such, similar to with topographical platforms, thousands of unique polymer–cell interactions are possible. These platforms have provided a wealth of information on how these cues can affect cell behavior.

Nevertheless, an important disadvantage of these platforms is that they only investigate one environmental type (either surface structures or chemistry), while cells in physiological environments are subjected to a wide range of stimulations. A consequence can be that combining surface structures and chemistry induces different or improved phenotypes compared to applying each perturbation individually. Therefore, a next generation of platforms that harness combinations of environmental perturbations in a high-throughput format is becoming more popular. An example of such a platform is the ChemoTopoChip.⁹ In this platform, 36 topographical structures and 28 chemistries lead to 1008 combinations, which can be harnessed for investigating cell behavior. The authors demonstrated that this platform allowed determining optimal combinations that induce alkaline phosphatase expression in MSCs, a marker for

Continued

State-of-the-art experiment—continued

osteogenesis. Furthermore, a different subset of material combinations was found to promote M2 macrophage polarization, which can be helpful for osteogenic differentiation of MSCs.

In the future, we will likely see increased development of such multiparameter platforms leading to more applications in the tissue engineering field (Fig. 8.17).

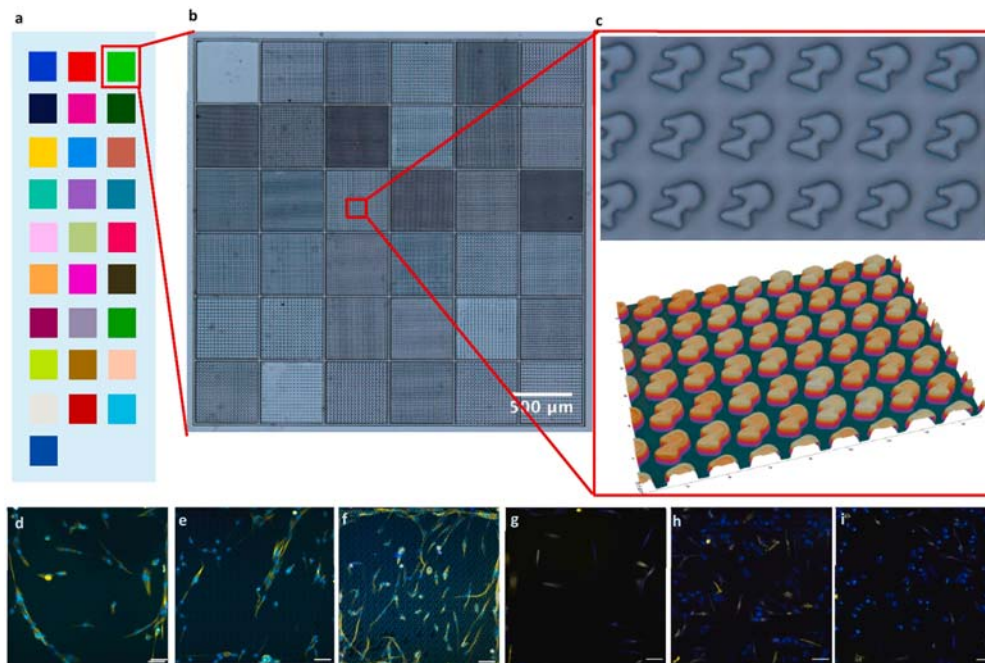


FIGURE 8.17

(a) Schematic showing ChemoTopoChip layout with colors representing different chemistries. (b–c) Example features. (d–i) Immunofluorescence of MSCs and macrophages. *From Burroughs et al., 2021. Discovery of synergistic material-topography combinations to achieve immunomodulatory osteoinductive biomaterials using a novel in vitro screening method: The ChemoTopoChip. Biomaterials. 2021;271:120740.*

8.7 Recommended literature

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8.8 Assessment of your knowledge

- a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
 1. What events lead to cell–material adhesion formation?
 2. What structures do cells use to probe their external environment?
 3. Describe and provide an example of mechanotransduction.
 4. Which major chemical properties of a substrate determine cell attachment and behavior?
 5. Which platforms exist to change cell shape?
 6. What consequences can changing cell shape have on cell behavior?
 7. How can hydrophobic and hydrophilic properties of a material be harnessed to control cell attachment?
 8. Why is the chemistry of a biomaterial important for clinical applications?
 9. What is the difference between static and dynamic chemistries on a substrate?
 10. How does material stiffness affect cell adhesion?
 11. What are the benefits of using synthetic polymer systems compared to naturally derived materials?
 12. How can the molecular clutch be used to explain cell response to material stiffness?
 13. Describe cell contact guidance.
 14. How does material stiffness effect MSC differentiation?

15. How have materials with controlled ligand spacing provided important insight into adhesion formation?
 16. Provide an example of integrin inside-out signaling.
 17. Provide an example of integrin outside-in signaling.
 18. Describe the molecular components of FAs.
 19. Explain how materials with low stiffnesses can lead to adipogenic differentiation of MSCs?
 20. Medical devices or implants can often fail due to the foreign body response, provide examples of how tissue engineering can be used to improve device/implant success.
- b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical and scientific situations:
1. How can tissue engineering strategies be used to control cell–material interactions?
 2. How do integrins provide mechanical linkages from materials to the nucleus, and what effects can this have on gene expression?
 3. How do we determine which chemical and/or topological cues are most important for controlling cell behavior?
 4. What would be the ideal properties of a biomaterial for wound healing?
 5. Would we be able to apply dynamic chemistry to create topological cues?
 6. What are the important parameters to consider when using topography for clinical applications?
 7. What tissue engineering strategies could assist you in developing successful implantable medical devices?
 8. What would be the ideal properties of a biomaterial for bone tissue engineering?
 9. Give an example of how we can use biomaterial systems as tools to investigate cellular processes or mechanisms.
 10. How could tissue engineered systems be used to control maintenance of stem cell pluripotency?

Challenge-based learning

A polymer patch to repair the contractile function in the infarcted heart

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Myocardial infarction (MI) is a high incidence disease worldwide. MI is the result of coronary obstruction and loss of blood flow to the cardiac tissue. Since clinical and surgical procedures cannot readily repair the fibrotic tissue, MI can lead to permanent tissue damage due to ischemia and loss of electrical conduction across the infarcted cardiac tissue. The long-term goal of this research is to generate materials to be deployed noninvasively that can readily restore electrical conduction and contractile force in the heart.

Motivation and stakeholders

The design space of polymers is enormous and polymer chemistry has yielded many interesting products. For instance, polymer chemistry can be tuned such that some materials can transduce electrical signals and others can show very high adhesive properties in watery conditions. Injectable and in situ gelling materials have been produced as well. Therefore, the state-of-art knowledge in material science and biomaterials can offer opportunities to generate conductive, adhesive, and injectable polymers with intended uses in MI. Solutions to mitigate this problem should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as patients at risk to suffer MI, cardiothoracic surgeons, cardiologists, and biomaterial engineers.

Challenge-based learning—continued

Problem definition

No single polymer formulation exists that combines all the requirements stated above to produce patches that conduct electrical signals across cardiac infarcts. There is a need to generate materials that are contractile, conductive, adhesive in watery conditions, as well as injectable to resolve the fibrotic, and often necrotic, cardiac patch after MI. This material should also facilitate the invasion and population of native cardiomyocytes to guide cell—material interactions toward the healing and regenerative route.

Challenge

To develop an injectable scaffold with conductive and adhesive capacities to provide a contractile and biocompatible bridge between the damaged myocardium and the healthy cardiac surroundings following MI.

Learning framework

Reading the Cell—Material Interaction and Principles of Cardiovascular Tissue Engineering chapters, and additional relevant literature will help you to understand the following:

1. The basic characteristics of a biomaterial when used as a scaffold for tissue regeneration.
2. The mechanisms of electrical conduction in the heart.
3. The ideal properties of a biomaterial used for myocardium regeneration.

4. The characteristics of hydrogels and their potential application in tissue regeneration strategies.

For a more focused examination of the challenge, create one mind map to include information about the following:

5. The challenges in designing a biomaterial appropriate for cell therapy in MI
6. The characteristics of conductive polymers and their potential to be used as biomaterials following MI.
7. The capacity of a conductive polymer to impact cardiomyocyte differentiation, specifically, in terms of electrophysiology and electrical signal transduction in the heart.
8. The challenges associated with designing biomaterials using conductive polymers.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution. More information and examples of CBL videos can be found in the student guide at www.jandeboerlab.com/TissueEngineering.

© Jan de Boer. CBL available for classroom use and can be found at www.jandeboerlab.com/TissueEngineering.

8.9 Glossary

Bidirectional signaling is the transmission of information in both directions across the plasma membrane.

Biocompatibility is the function of a material to trigger an appropriate host response without causing undesired local and systemic effects.

Cellular adhesion is the process by which cells form contacts with each other or with their substrate through specialized protein complexes.

ChemoTopoChip is a novel combinatorial chemistry—topography screening platform to assess the suitability of biomaterials to be used in tissue engineering.

Contact guidance refers to a phenomenon for which the orientation of cells and stress fibers is influenced by geometrical patterns such as nano/microgrooves on substrates, or collagen fibers in gels and soft tissues.

Cross-reactivity is the binding of a (protein) molecule to chemically or structurally similar ligands to the cognate ligand.

Dynamic chemistry is when surface chemistry of a material is triggered to change in response to stimuli such as light and changes in pH or temperature.

Focal adhesion kinase is a signaling protein in the focal adhesion complex

Focal adhesions are large macromolecular assemblies through which mechanical force and regulatory signals are transmitted between the extracellular matrix and an interacting cell.

Focal complexes are a collection of integrin adhesion complexes in a cluster at an adhesion site, typically found at the cell front.

Hydrophilic(ity) is the physical property of a molecule or surface that is seemingly attracted to a mass of water.

Hydrophobic(ity) is the physical property of a molecule or surface that is seemingly repelled from a mass of water.

Inside-out signaling is when intracellular signals act on integrin cytoplasmic domains, inducing conformational changes in integrin extracellular domains, resulting in increased affinity for ligand at the cell surface.

Integrin adhesion complex (IAC) is a protein complex formed by direct linkage of extracellular matrix, integrins, the cytoskeleton, and associated signaling/structural proteins.

Integrin clustering is the assembly of several integrins at the plasma membrane.

Intracellular tension is the force produced by contraction of actin and myosin-based cytoskeleton.

Ligand spacing is the physical space between two ECM-bound ligands.

Mechanobiology is a field of study that focuses on how physical forces and changes in the mechanical properties of cells and tissues contribute to development, cell differentiation, physiology, and disease.

Mechanotransduction is the processes through which cells sense and respond to mechanical stimuli by converting them to biochemical signals.

Medical devices are any device intended to be used for medical purposes.

Microenvironments are comprised of micro and nanoscale properties of distinctly specialized and effectively isolated biophysical environments.

Motifs are specific combinations of amino acids in proteins that form a functional unit.

Outside-in signaling occurs when an extracellular ligand binds to the extracellular domain of a protein and initiates intracellular signaling events.

Self-assembled monolayers are molecular assemblies formed spontaneously on surfaces by adsorption and are organized into smaller or larger ordered domains.

Shear forces are mechanical frictional forces, parallel to the blood flow exerted on the endothelial wall of the vessel. These forces are maximum at the inner surfaces of a vessel and almost zero at the center of the blood vessel.

Solid-state interactions are the interactions between the structure and chemistry of a material and cells/tissue defined. These interactions can confer effects through physical distortion, chemical signaling, adhesion points, or a combination of these.

Surface roughness is a component of surface texture quantified by the deviations in the direction of the normal vector of a surface from its ideal form. If these deviations are large, the surface is rough; if they are small, the surface is smooth.

Topography is the architectural landscape of the surface of a (bio)material.

Traction forces are mechanical forces exerted by the cells to perform various tasks, including maintaining cell shape, migrating within tissues, reorganizing ECM, and communicating with neighboring cells.

Water contact angles are the angles formed when the water interface meets a solid surface. It is used to quantify the wettability of a solid surface.

Young's modulus (or the modulus of elasticity) is a mechanical property that measures the tensile or compressive stiffness of a solid material when the force is applied lengthwise.

“Click” chemistry is a class of biocompatible small molecule reactions commonly used in bioconjugation, allowing the joining of substrates of choice with specific biomolecules.

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Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Biomaterials discovery: experimental and computational approaches

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9.1 Learning objectives

After reading this chapter you will be able to:

- Explain the requirement for and difficulties of biomaterials discovery.
- Design an efficient experimental approach for biomaterials discovery.
- Appraise computational modeling within materials discovery projects.
- Relate the key components of a neural network.
- Describe the key steps associated with the development of a white box computational model.

Any problem can be solved using the materials in the room

E. Land.

The adoption of combinatorial and computational methods in biomaterials design is a highway towards the discovery and realization of tailored polymeric materials ...

J. Kohn.

9.2 Introduction

Current biomaterials have revolutionized aspects of medicine. However, they still routinely fail in the clinic due to immune rejection, infection, mechanical failure, or because they require debilitating drug regimens such as blood thinners to prevent clotting. The poor biological performance of medical devices is because materials used in these applications were often chosen from existing, “off-the-shelf” materials with suitable mechanical properties, but with little or no regard to how biology interacts with these materials. For example, silicone rubbers were initially developed as electrical insulators not as urinary catheters, yet millions of such devices are used in healthcare today. While silicone rubbers have many useful properties such as flexibility, low cost, and chemical resistance, their surface properties are far from ideal, as they support high rates of catheter associated urinary tract infections. Similarly, stents used to hold blood vessels open are typically made from stainless steel that induces blood clotting. As a result, patients with stents are required to take anticoagulants for the rest of their lives. Clearly, using existing materials for the rapidly increasing range of biomedical applications is simply not good

enough as it compromises the effectiveness of existing materials and devices, and hinders development of new medical applications.

Tissue engineering continuously requires new biomaterials to create therapies based on cellular delivery or organ (re)generation. Within **regenerative medicine**, there is a strong drive to translate prospective therapies into the clinic (see [Chapter 21 Clinical translation](#)). Often this means that choice of materials is dominated by materials, such as poly(lactic acid) and poly(lactide-co-glycolide), already licensed for use in humans. However, these materials are often poorly suited to a particular cell type or desired cellular response, so it is essential to have the ability to develop new materials with properties tailored for a particular need (see [Chapters 5, 6 and 7 on various aspects of biomaterials engineering](#)).

9.3 The challenges of biomaterials discovery

Materials science is a well-established discipline that has been, and continues to be, an area of extensive research activity. It can be characterized as the study of how a material's structure determines its properties (**structure—function relationship**) and how this understanding can be used to achieve desirable performance through appropriate molecular design and processing/synthesis. For example, the mechanical properties of a metal used in the construction of an aero plane can be controlled by manipulating its microstructure using appropriate heating, cooling, and mechanical deformation. This process has been practiced since the formation of the first wrought iron items, such as nails, by blacksmiths through to advanced aerospace alloys. Composite materials then developed slowly over centuries to provide the current toolkit of high-performance structural materials. The accumulated knowledge in these material systems now allows an aerospace engineer to “dial up” the material composition, sample preparation, and product shape from a database to design entire aircraft or individual components on a computer ([Fig. 9.1](#)).

The level of knowledge required to achieve the same situation for biomaterials discovery ([Table 9.1](#)) has not yet been developed ([Fig. 9.1](#)) as it requires additional dimensions of understanding. Whereas many materials used in nonbiological applications can have their properties and working environments modeled and predicted by physical laws, the laws of biological interactions are less defined and **biological environments** are more complex. For example, to engineer a material that can act as a scaffold to support cell growth we must not only consider physical and mechanical property requirements but also how cells interact with materials (see [Chapter 8 Cell-material interactions](#)). Thus, we must also

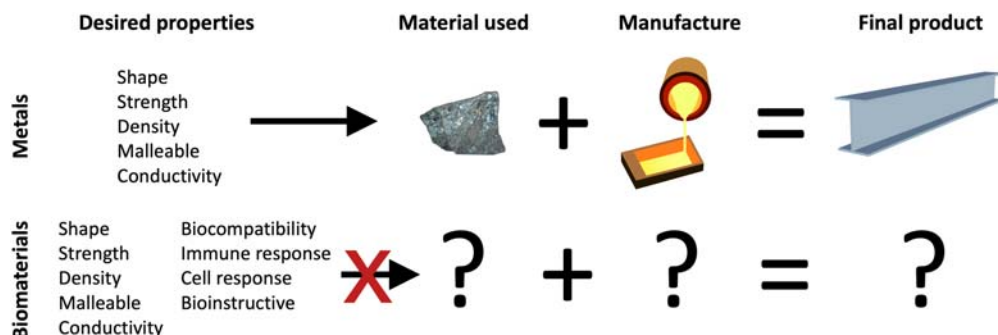


FIGURE 9.1

Comparing the structure—property knowledge between mechanical engineering and biomaterials.

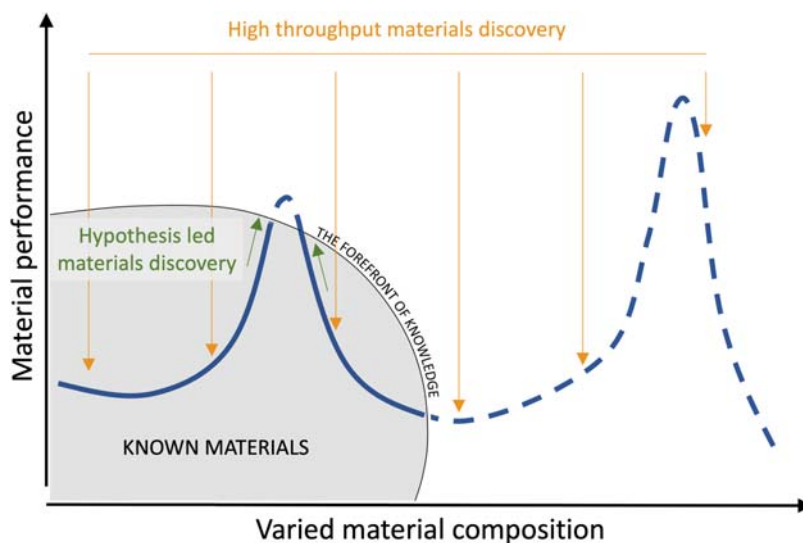
Table 9.1 A list of key terminology associated with biomaterials discovery.

The terminology	Definition
Biomaterials	A synthetic or biologically derived material for application in biology
Combinatorial space	A range of materials created by combining a smaller number of base components
Computational modeling	Using statistical, machine learning, or equation-based methods to simulate and predict the behavior of materials and biological systems
High throughput screening	A method of experimentation involving automated parallelization of experimental procedures, such that many tests can be conducted in the time it takes to perform a single test conventionally
Hit material	A member selected from a library of materials that has superior performance
Materials discovery	Screening a materials space (often a combinatorial space) to discover a material optimal for a particular application
Materials or chemical space	The set of all materials or chemistries that exist or could theoretically be produced
Mathematical descriptor	A mathematically entity that encodes various properties of a material
Ontologies	Controlled vocabularies that precisely describe, in terms computers can understand, multiple aspects of materials such as composition, synthesis, processing, and properties
Polymer library	A collection of different polymers
Polymer microarray	An experimental platform that allows hundreds to thousands of unique materials to be expressed on one support, e.g., a microscope slide, at addressable locations
Provenance (as related to materials)	The history of a medical device related to how it was made, how it was processed, and conditions the materials have been exposed to
Synergistic effects	Whereby a combination of components produces a nonadditive property that could not be predicted from assessing the properties of the individual components

consider **biocompatibility**, **biodegradation**, the laying down of extracellular matrices or biofilms, cellular adhesion, immune compatibility, cell adhesion density, and changes to **cell phenotype** such as cell shape, marker expression, fate, and other behaviors. Current understanding of these interactions is not sufficiently detailed to enable a researcher to “dial up” the appropriate materials for a device that, for example, manipulates cells into a vascularized functioning organ.

9.4 Approaches to materials discovery

Searches for new materials begin with experimental observations of the performance of a set of candidate materials. In some cases, there is sufficient knowledge of the biological response to allow hypothesis-led materials discovery (Fig. 9.2). This is achieved by expanding the knowledge about

**FIGURE 9.2**

Comparing hypothesis led and high throughput materials discovery.

materials through postulating and testing hypotheses based upon prior knowledge. A good example of this approach is poly(ethylene glycol) (PEG). This polymer is well known for its ability to prevent protein adsorption (**nonfouling**). This polymer was optimized by developing syntheses for creation of dense polymer brushes that were particularly effective at preventing biomolecule adsorption. Numerous innovative methods were developed to coat the surfaces of medical devices with PEG brush layers. As a result, this polymer has been successfully deployed in many different application areas including cell scaffolds, drug delivery systems, diagnostics, and wound healing.

However, PEG is almost certainly not the very best nonfouling polymer, indeed its **self-oxidation** properties limit its long-term usage. Hypothesis-led research has a limited ability to extend prior knowledge to find novel materials. For biomaterials discovery, where the interactions are complex and understanding is limited, another approach is required.

Surprisingly, a “blind” approach to materials discovery can be useful in cases where the biology–materials interaction is not well understood. It enables discovery of entirely new classes of materials that could not have been predicted from the current knowledge of a given biological response to a material. The success of this approach depends on the *quality* (how well the materials and the biological measurements approximate the final intended use of the materials, and reproducibility of the measurements), *quantity* (the total number of measurements), and *diversity* (how much of the total **chemical design space** is represented by the materials being studied) of the experimental measurements on the initial candidate materials. Given the size of accessible **materials design space** (see below), the likely success of a blind approach to materials discovery is clearly very low. It is, therefore, necessary to screen as many measurements as possible. To achieve this, high throughput materials discovery approaches have been developed where hundreds to thousands of different materials are screened in

parallel. These have recently been augmented and greatly extended by the application of computational modeling methods to discovery and optimization of biomedical materials, and materials generally.

9.5 Experimental high throughput materials discovery

To perform a high throughput screening, hundreds to thousands of materials need to be synthesized and presented in a way that allows meaningful measurements to be taken rapidly and automatically. The format in which this is achieved can vary, but there are several key principles that always need to be considered. This is explored through the case study of the polymer microarray (Fig. 9.3). In this format, small polymer spots are printed onto a solid substrate at addressable locations. The design of a polymer microarray is directed by the **biological assay** used, and consideration must be given to the following:

1. the supporting substrate and its coating,
2. the members of the polymer library and their synthesis
3. the biological assay that is coupled to the microarray (Fig. 9.3).

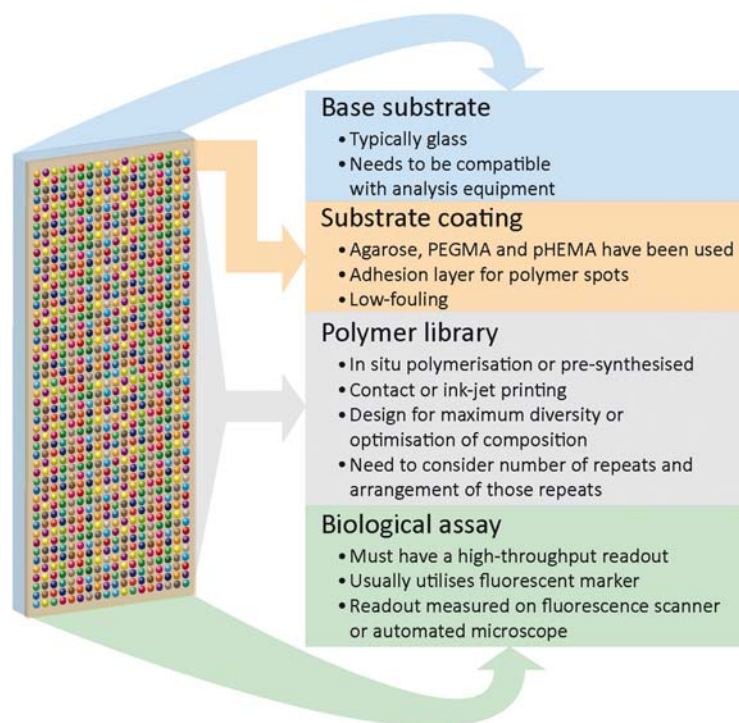


FIGURE 9.3

The key parameters that must be considered for the creation of a polymer microarray. *From Hook AL. Polymer microarrays for high throughput biomaterials discovery. In: Palmer E, ed. Cell-Based Microarrays—Review of Applications, Developments and Technological Advances. New York, USA: Springer; 2014.*

9.5.1 The supporting substrate and coating

The main requirement for the array support is that it can be employed easily in the biological assay and associated high-throughput readout. For polymer microarrays, the substrate of choice is a glass microscope slide due to the existing range of stage holders, scanners, and microscopes that accept this format. Furthermore, the slide is transparent, allowing cells growing on the slide to be easily assessed using light microscopy. The slide is also widely compatible with most cell culture methods, enabling a diverse range of biological assays to be used. However, polymer microarrays can also be produced on other materials, for example, onto polystyrene-based tissue culture ware that enables the microarray format to be used in a larger number of biological assays.

The surface chemistry of the underlying substrate plays an important role in the formation of a microarray, as well as the success of subsequent bioassays. The substrate coating must be both adherent to the materials printed onto it and resistant to the attachment of biomolecules and living cells (nonfouling), to optimize the signal to noise ratio of any biological assay and prevent cross-talk between spots. Several different surface coatings have been developed to address these needs, with priority being given to cheap and robust coating methodologies. Examples include poly(hydroxyethyl methacrylate)-, agarose-, and poly(ethylene glycol) methacrylate-based coatings.

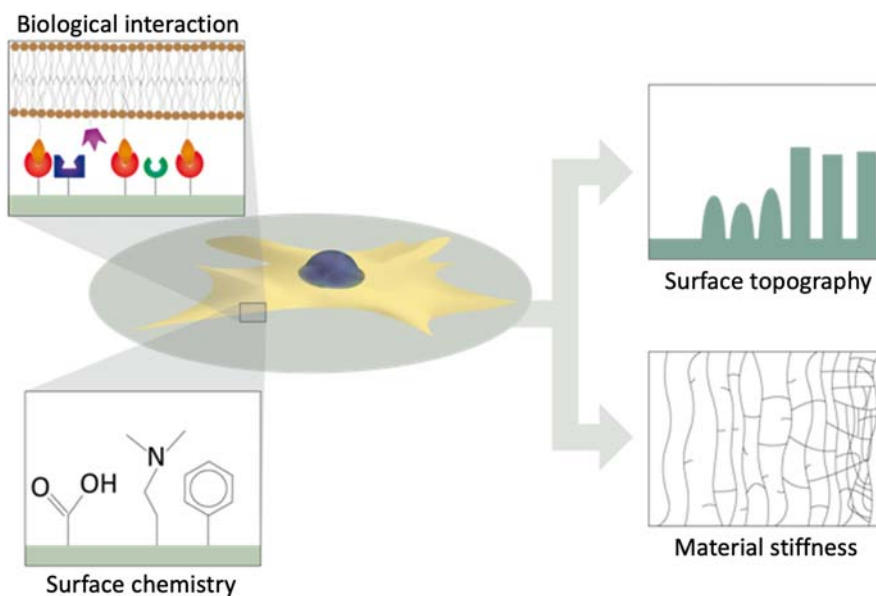
9.5.2 Materials libraries

Creation of material arrays requires a library of materials to be generated and positioned at addressable locations on a substrate surface. Materials used on a polymer microarray will be determined by the biological–material interactions being probed. It is possible to systematically explore chemistry and other contributors to the cell response to materials. These are broadly collected into four groups: specific biochemical ligand interactions (e.g., surface peptides); surface chemistry; surface topography; and materials mechanical properties (e.g., stiffness) (Fig. 9.4). Some or all of these properties may be varied on an array depending on the biological response to materials under study. The ultimate screen of a biological–materials interaction would include all relevant materials properties.

9.5.2.1 High throughput screening systems

Synthetic polymers, the focus of this chapter, have been broadly used as candidates for biomaterials as their composition can be controlled easily in most cases, they are relatively cheap, and are amenable to large scale manufacture. Creating a library of materials requires large numbers of materials to be synthesized quickly. Polymer families such as polyacrylates and polyacrylamides (polymerized using a free radical mechanism) are particularly suitable as they polymerize quickly and there are many different monomers available commercially.

High throughput screens of material–cell interactions are not limited to synthetic polymers but can be applied to **biological molecules** such as proteins. The proteins of greatest interest are those whose biological function is associated with cell attachment. Within the body, cells secrete and bind to an extracellular matrix (ECM) composed of polysaccharides and proteins, such as collagen, laminin, vitronectin, and fibronectin (Fn), that modulate cell behavior (see [Chapter 5 Extracellular matrix as a bioscaffold for tissue engineering](#)). Individual proteins and their combination can be printed directly onto glass substrates to form a high throughput platform that can be used to assess the response

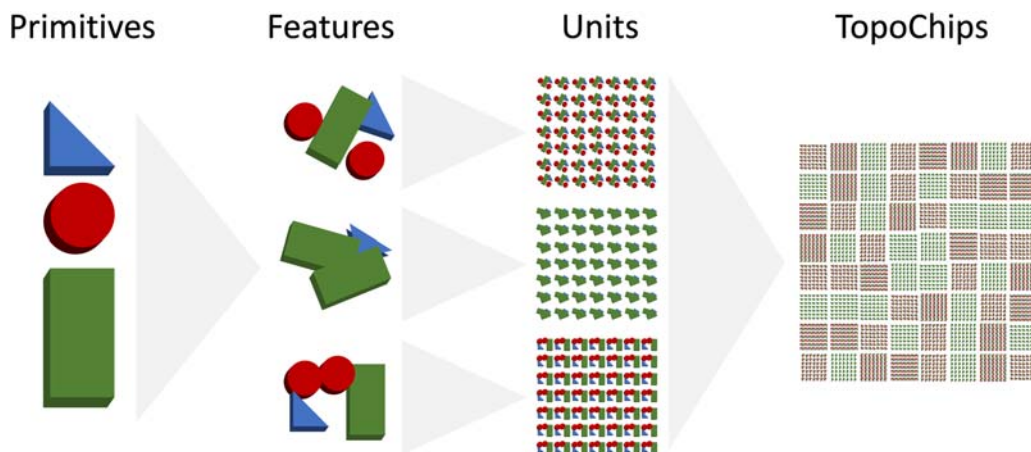
**FIGURE 9.4**

The key aspects of biological–material interactions. From Hook AL. *Polymer microarrays for high throughput biomaterials discovery*. In: Palmer E, ed. *Cell-Based Microarrays—Review of Applications, Developments and Technological Advances*. New York, USA: Springer; 2014.

of cells. One study used this approach to discover that a combination of collagen and Fn was optimal to drive the differentiation of embryonic stem (ES) cells to an early hepatic (liver) fate. This observation was then used to design a biomaterial with improved ability to instruct ES cell differentiation.

The **topography** (roughness or intentional creation of micro- or nanoscale shapes) of a surface plays an important role in the attachment and behavior of cells. For example, contact guidance of cells along grooves has been observed down to nanoscale feature sizes, altering the roughness of prosthetic implants has been a key factor in improving bone-implant contact, and it has been demonstrated that surface roughness influences differentiation of preosteoblast (bone) cells. Thus, surface topography is a key property to be included in biomaterial development programs.

By combining different shapes, angles, curvatures, spacings, and feature densities, the number of resulting topographies that can be generated are endless. Thus, the challenge is to produce a subset of materials that is representative of the materials' topography space. In one strategy (Fig. 9.5), topographical features were derived from three primitive geometric shapes—circles, triangles, and rectangles arranged within an imaginary square. The three base shapes are good representative base units as they allow the inclusion of large smooth areas (circles), angles (triangles), and stretched elements (rectangles) within the resultant topographical features. Each topography was replicated many times to create units with sufficient area to allow cells to interact with them to provide statistically significant cell response measurements. Although this approach provides an extremely large number of possible topographies, one

**FIGURE 9.5**

Design of a TopoChip. From Unadkat et al. *An algorithm-based topographical biomaterials library to instruct cell fate*. Proc Natl Acad Sci USA. 2011;108:1,6565–16,570.

study only used 2176 units, selected at random, and generated by photolithography and embossing of poly(lactic acid) films to produce a TopoChip (Fig. 9.5). TopoChips have been used to study the influence of topography on a number of different biological systems including the **bioactivity** of human mesenchymal stem cells. Varying levels of alkaline phosphatase, a marker for early osteogenic differentiation, were observed for cells grown on the different topographical patterns. This demonstrated that surface topographies alone can induce targeted cellular differentiation.

High throughput screens of **material stiffness** are typically achieved by the creation of hydrogels, such as polydimethylsiloxane, polyacrylamide, or PEG-based gels, where the modulus of the material can be varied by altering the cross-linking density. Use of a gradient system, where a sample is produced that varies continuously from a low to high stiffness, is commonly used to enable a range of material properties to be screened at the same time. These systems have played an important role in determining how substrate stiffness influences stem cell differentiation (see [Chapter 8 Cell–material interactions](#)). A key consideration when designing these systems is to ensure that varying one material property does not alter another property. For example, increasing cross-linking density may also result in a change in surface chemistry. It is necessary to analyze multiple surface properties of high throughput systems to identify which properties are involved in the resultant biological response.

Although relevant to any cell–material interaction, it is particularly important to study the **mechanical interaction** between cell and materials within a 3D cell culture system (see [Chapter 11 Scaffold design and fabrication](#)) in order to better mimic the in vivo environment where mechanical stresses occur in all directions around a cell. This has been achieved by first loading a gel with cells and then applying stress by compression or stretching. Use of microfluidic systems or asymmetric stretching has been used to apply different strains across samples to explore cell–material interactions in high throughput.

9.5.2.2 Combinatorial polymer libraries

Large numbers of materials can be synthesized quickly using a combinatorial approach, whereby a small number of base components are combined at varied ratios to produce many materials. For polymers, the combinatorial reaction of a small number of monomers produces many copolymers for screening. This is similar to the topography example (Fig. 9.5), where a small number of primitive units can be combined to create a large and diverse set of surface topographies. Combinatorial polymer libraries may be created by simple physical mixing of different ratios of monomers to produce a library of copolymers. For example, 24 different monomers can be mixed pairwise at a 1:2 ratio to produce $24 \times 24 = 576$ unique polymers. Larger numbers of polymers could be synthesized by using three monomers, or by mixing monomers with a larger number of ratios. The chemical diversity of the polymer library is limited by the original 24 monomers used, but such a library allows exploration of the chemical space with greater resolution. These libraries are also ideally suited to training statistical and machine learning models discussed below.

9.5.2.3 Genetic methods for materials design

To overcome the limited range of materials space probed by combinatorial libraries, scientists are also exploring genetic methods for materials design. These methods resemble Darwinian evolution where organisms compete for resources in particular environments, and the only fittest survive to thrive and reproduce. In this approach, a family of potential materials exhibiting the desired property are synthesized and tested. The materials that have the best performance in relevant tests are identified, and modified version of them synthesized for testing in the next round of evolution. Close analogues of the best of these are again synthesized and tested. This process is continued for several rounds of evolution until suitable materials are identified or no further improvement is observed.

The advantage of **genetic methods** is that they can, in principle, efficiently explore very large materials spaces to identify regions containing useful properties. As distinct from combinatorial screening, which aims to generate a large number of materials quickly (Fig. 9.6, if different colors represent different chemistries, combinatorial screens rapidly generate lots of materials composed of the same base

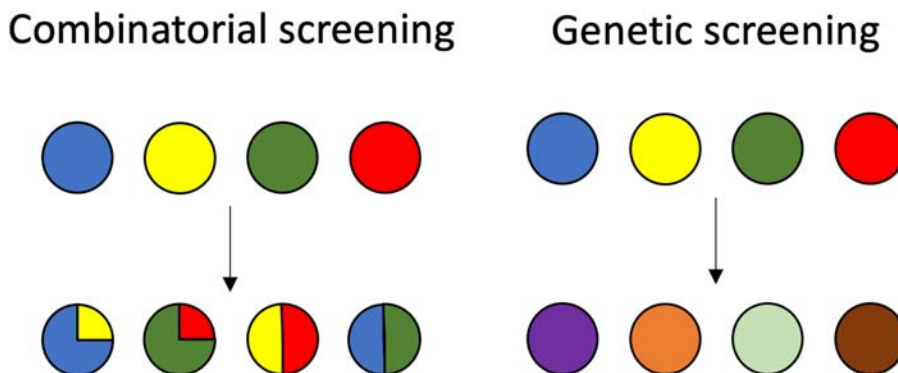
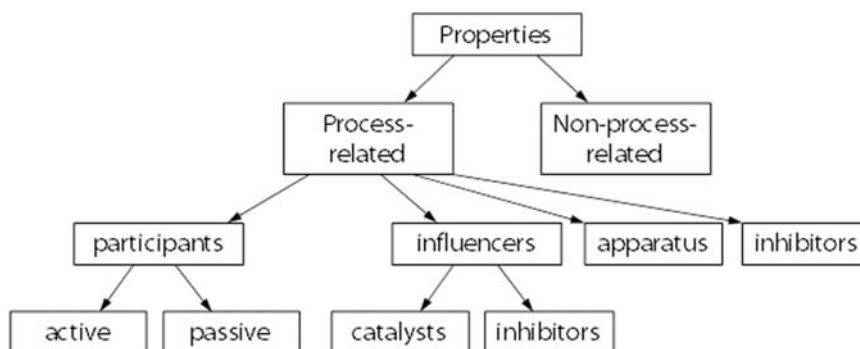


FIGURE 9.6

Comparing combinatorial and genetic screening.

**FIGURE 9.7**

Schematic of a multigeneration screening approach.

colors), genetic methods will arrive at an optimal material that is different from those that were in the initial screen (Fig. 9.6, genetic methods result in new colors of materials). However, they may not find the very best possible material in the entire space of possibilities but, like Darwinian evolution, find materials that have **locally optimal properties** that are, however, novel and have an excellent performance.

A simple example of the genetic method is a multigeneration screening approach (Fig. 9.7). By design, the first-generation screen has the largest variation in composition but does not probe any particular composition in detail, i.e., chemical diversity is high, but chemical resolution is low. Subsequent generations of synthesis and screening are generated by mutating the genomes of the fittest materials from previous generations, resulting in gradual reduction in **chemical diversity** but increases in **chemical resolution**. As an example, 10 initial chemically diverse monomers are used (indicated by color) (Fig. 9.7). The three fittest materials were selected from the first-generation screen and mixed pairwise to form a 9-member combinatorial polymer library as a second-generation array. The best performing pair was selected for the final generation screen in which the two materials are used to make a series of copolymers with small changes in the ratio of the two components. The inclusion of the same materials (**homopolymers**) in each generation is important for comparing and cross-validating the results from each generation.

9.5.2.4 design of experiments

The major bottleneck of material discovery studies is the need to synthesize and experimentally test a large number of materials. As the size of materials spaces is so large, it is important to design experiments so that the smallest number of experiments can cover as much of the materials space as possible and provide the experimentalist with as much information as possible. **Design of Experiments (DoE)** allows experimental scientists to carry out a relatively small number of materials syntheses or characterization experiments that can be representative of a much larger number of materials. This makes optimal use of often expensive, and time- and resource-consuming experiments. This approach is used in many different fields and is clearly relevant to biomaterials discovery. The method involves sampling specified regions of materials or design space with various degrees of sparsity. For example,

if there are three experimental parameters, X_1 , X_2 , and X_3 , that can be varied, and they are used at 10 different levels, there are $3^{10} = 59,049$ experiments required to completely describe the space. This is normally intractable except with very high throughput methods.

As an alternative, **full factorial design** assesses the main effects within a system by considering combinations of each experimental parameter, such as structural/compositional properties, synthesis, and process parameters, reduced to discrete values (e.g., high/low, polar/nonpolar, negative/positive). The experiments selected for a full factorial design (Fig. 9.8, shown as the vertices) enable the effects of each individual parameter, each pair of parameters and the combination of all three parameters to be explored using a smaller number of experiments. For example, if three parameters (k) each take two levels (l) there are $l^k = 2^3 = 8$ experiments (the vertices of Fig. 9.8), a significant reduction from 59,049. However, if the number of parameters increased to 7 and the number of levels to 3 (e.g., negative/neutral/positive) there are $l^k = 3^7$ or 2187 experiments if all the possible combinations of these parameters are explored experimentally. Again, this number of experiments is too costly or time consuming.

A **partial factorial design** allows conduct of a smaller number of experiments l^{k-P} depending on the parameter P , the size of the fraction of the full factorial design used. This essentially means that the materials or design space is sampled more sparsely. As we will see later, computational models can be trained with these data allowing the properties of the entire space to be predicted. The principle is illustrated by a simple example (Fig. 9.8). A full factorial design is represented by the eight spheres at the vertices of the cube. If each factor could take either of two values, there would be $2^3 = 8$ possible experiments. A partial factorial design with $P = 1$ would require $2^{3-1} = 4$ experiments. If the experiments

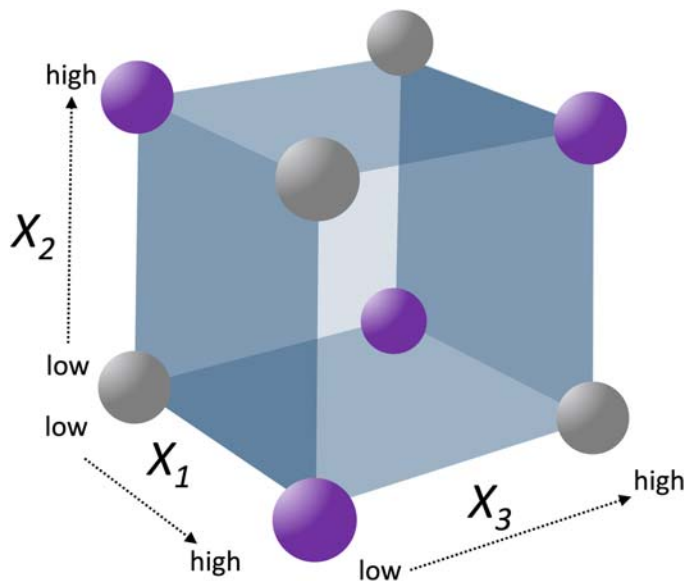


FIGURE 9.8

Schematic depiction of design of experiments.

were correctly chosen, each factor would occur at the high and low levels the same number of times (balanced design). As an example, all the purple spheres or the gray spheres (Fig. 9.8) would represent a balanced partial factorial design.

Mathematical or statistical models can then make predictions of the properties of the materials that were not synthesized or tested in the partial factorial design, that is, the models can “fill in the gaps.” These or existing experiments on materials, particularly those done using high throughput robotic methods that generate large amounts of data, can be used to generate sophisticated mathematical models that relate material composition, structure, processing, etc., to their performance to modulate cell activity as biomaterials, tissue engineering scaffolds, or solution borne molecules. This enables the most to be learnt from a small number of experiments that can represent that largest possible materials space.

9.5.2.5 Synergistic effects

High throughput screens have made clearer the incidence of **synergistic effects** (Fig. 9.9), where the combination of two components induce a biological response that would not be predicted by assessing the response to each component alone. For example, the performance of a synergistic two-component system will out-perform the two respective one-component systems. The occurrence of such synergies is an important advantage of combinatorial materials library design and also highlights the need for greater use of high throughput assays to uncover these unique and unpredictable interactions.

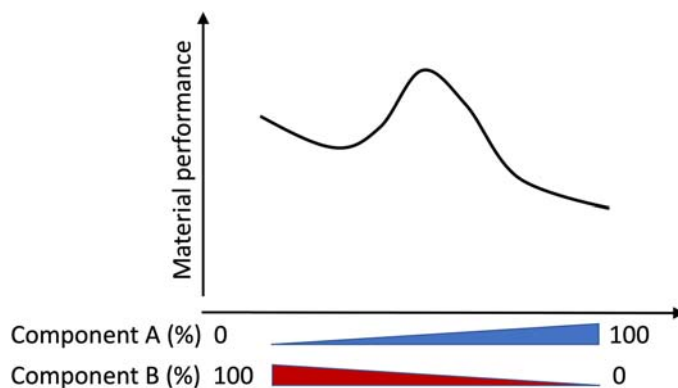


FIGURE 9.9

Example of the observation of synergistic effects observed in a two-component system.

9.5.3 Biological assays

The final consideration for applying polymer microarrays to the discovery of biomaterials is the design of bioassays that are compatible with the high throughput format. This requires a relevant **readout** that can be measured in an automated fashion. Typically, fluorescence markers are used that can be read using an automated fluorescence microscope or a fluorescence scanner. Assays must be fast and relatively inexpensive. Each biological application requires the development of a suitable biological assay that is specialized for each case. Polymer microarrays have been successfully used to probe a diverse

range of biological systems, including supporting stem cell growth and directing differentiation, instructing the behavior of macrophages to pro- or antiinflammatory phenotypes, dendritic cell maturation and phagocytosis, materials resistant to bacteria, switchable materials, platelet activation, cell sorting, hepatocyte and cell toxicity models, human skeletal cell attachment, endothelization, giardia lamblia material interactions, cell transfection and *Cryptosporidium parvum* material interactions. The large number of biological systems that has been successfully assessed using high throughput screening platforms demonstrates the versatility and platform nature of this approach.

Typical high throughput materials discovery projects begin with a materials library that is as large and diverse as possible. At this stage, there will be inevitable compromises in the biological assays to minimize cost and researcher time. The biological assay that is most informative for materials intended for medical device applications is a human trial. Clearly, there are high ethical barriers and time and financial costs involved with high throughput human trial, especially where >99% of experiments may fail. Hence, initial screens are chosen to be in vitro biological assays that ideally are indicative of in vivo responses. However, they often overly simplify the system being studied and can be susceptible to **false negative** (missing potential hits) or **false positive** (selecting materials that are not really hits) selections. It is necessary to ensure thresholds used to select hits are not overly stringent in the initial screens, resulting in few hits, and that subsequent assays are conducted to verify and validate hits (Fig. 9.10).

One example of this is the use of polymer microarrays to identify materials that instruct macrophages to become either pro- or antiinflammatory. The change in phenotype is highly complex and can be described by cell-surface biomarkers, excreted small molecules (cytokines), and by the influence the

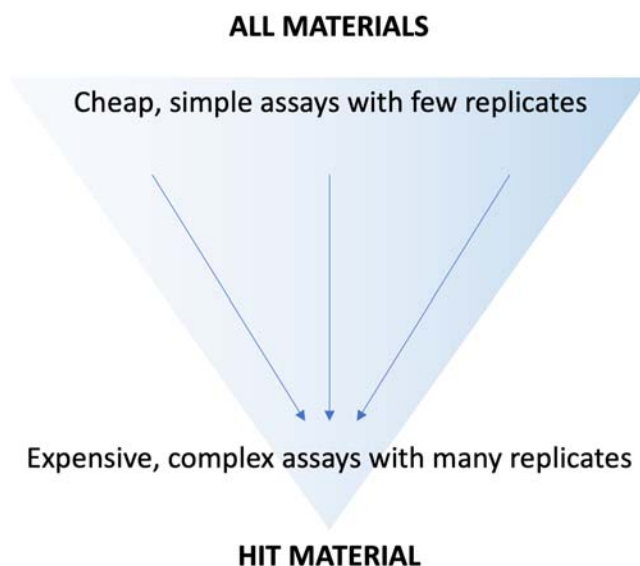


FIGURE 9.10

The changing requirements of biological assays in a materials discovery project with the progression toward a hit material.

cells have on the foreign body response in vivo. A high throughput screen with this system could be conducted in three stages:

1. Use a small quantity of a large number of materials with few replicates and assess a single biomarker for the pro- and antiinflammatory phenotypes as a low-cost assay.
2. Take hits from screen 1, create larger samples with more replicates, and assess more biomarkers and the excreted cytokines as a medium cost assay.
3. Test the most promising hits from screen 2 in vivo to assess the foreign body response as a high-cost assay that is most informative.

9.6 Computational materials discovery

9.6.1 Problems and opportunities raised by the size of chemical space

To paraphrase Star Trek, *Chemical Space: the final frontier, our mission is to explore strange new chemistries, to seek out new properties, to boldly go where no man has gone before* ... If we are to accept the challenge of exploring chemical space thoroughly, we cannot do this by experiment alone.

It has been estimated using the laws of chemical valence that 10^{100} different molecules could be synthesized with molecular weights below 1000 Da. Such a large number is difficult to comprehend; it is greater than the estimated number of particles of matter in the observable universe, approximately 10^{80} atoms! This creates problems in that all of materials space cannot ever be explored exhaustively. This also means there is potentially an almost infinite number of materials to discover, many of which will have greatly improved properties for a vast array of applications.

High throughput screening approaches can assay hundreds to thousands of samples in parallel. This is at least 100 times faster than conventional single material measurements but still vastly too slow to explore even a fraction of the possible materials that could be synthesized. The most advanced existing or future robotic systems for materials synthesis and property measurement cannot create more than a minute fraction of the materials that could theoretically be produced. Even if you could synthesize and characterize 1 billion materials a second for 1 billion years, you could only make $\sim 10^{25}$ materials. It is clear that we need a more efficient method for navigating enormous regions of molecular space. Given ever-increasing computational power and recent development of dramatically improved machine learning methods, we can describe and navigate these huge spaces in silico, making it feasible to explore and identify valuable regions of materials space many orders of magnitude faster than real experiments. The preceding section on DoE and the following sections on ontologies and modeling deal with these separate challenges, physical, and computational experimentation, respectively. Being data-driven methods, machine learning-based modeling methods are complementary to, and critically dependent on generation of data described in sections on high throughput experimentation. The large number of biology-materials interactions that can be assessed using high throughput screening methods provides an ideal basis for the training of models.

9.6.2 Introduction to computational modeling

Mathematical, statistical, and computational methods are being adopted to allow researchers to create “virtual materials” and computational experiments that can achieve useful outcomes. Computational

modeling provides the bridge between the experimental limitations and the vastness of chemical and materials spaces.

Materials scientists and tissue engineers are fortunate in that some of the required high throughput experimental methods and many of the mathematical modeling tools have been developed previously by the pharmaceutical industry. In the early 1990s, methods for rapid synthesis of potential drug candidates using combinatorial chemistry were developed. This allowed hundreds or thousands of new molecules to be made simultaneously instead of one at a time, as had been done previously. At the same time, very fast and efficient methods for measuring the biological effects of these large libraries of drugs were also developed.

This paradigm shift in the way drugs were discovered also increased the amount of data generated by orders of magnitude, requiring new methods of data processing, storage, and most importantly modeling to be developed. The skills developed in the pharmaceutical industry have been transferred to materials and tissue engineering, meaning that capabilities in high throughput discovery in this research domain are accelerating rapidly. The mathematical and statistical tools that were developed to make sense of the vast amount of data generated by the pharmaceutical industry are also very applicable to data generated by materials scientists and tissue engineers, with one important difference. The complex materials employed in materials science and tissue engineering are not single, precisely defined molecules like drugs. They are often complex polymers with distributions of chain length, often cross-linked in random ways, or containing mixtures (blocks) of multiple polymer segments derived from two or more monomers. They do not act chiefly at one or a few molecular targets as drugs often do; their interactions with biological systems are multifactorial and much more complex.

In tissue engineering specifically, biomaterials could be complex biological polymers with a mixture of components with distribution properties at the molecular scale. Complex materials often have properties, particularly surface properties that largely control cellular responses, which are sensitive to the way the materials are made and processed (their **provenance** or history). For example, a simple medical plaster consists of multiple components (an absorbent area, an adhesive area, a structural area) that are synthesized and then arranged in a very specific way in order to make the plaster function as intended. For biomaterials, the processing of materials can impact the molecular structure. Developing analytical methodologies to measure the actual surface chemistry and adapting computational modeling methods to deal with this heterogeneity of structure and provenance dependence is one of the most important research issues in biomaterials discovery.

Once the structural, **physicochemical**, and provenance properties of complex materials are described mathematically in the form of **descriptors**, it is possible to build very effective models relating these descriptors to biological responses to materials using statistical and machine learning methods. These methods of mathematically mapping physicochemical, structural, and provenance properties to biological responses or physical properties are known generically as **quantitative structure-property relationships** (QSPR) modeling methods. These mathematical relationships can be as simple as linear regression models relating the intrinsic molecular, structural, physical, and process properties of a material to some useful property that the material exhibits. In many cases, the relationships between the molecular or process properties and the useful overt properties of materials are very complex and nonlinear. In these instances, machine learning methods like neural networks can be very effective at

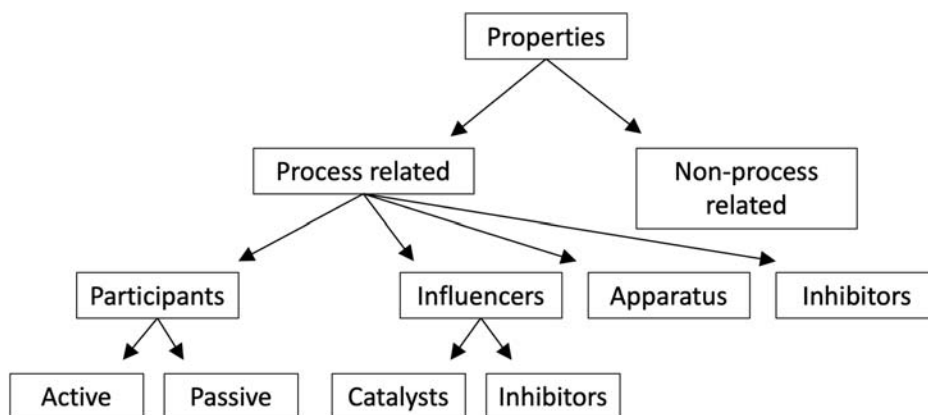
finding this complex relationship and making accurate predictions of useful properties of new materials.

9.6.3 Ontologies

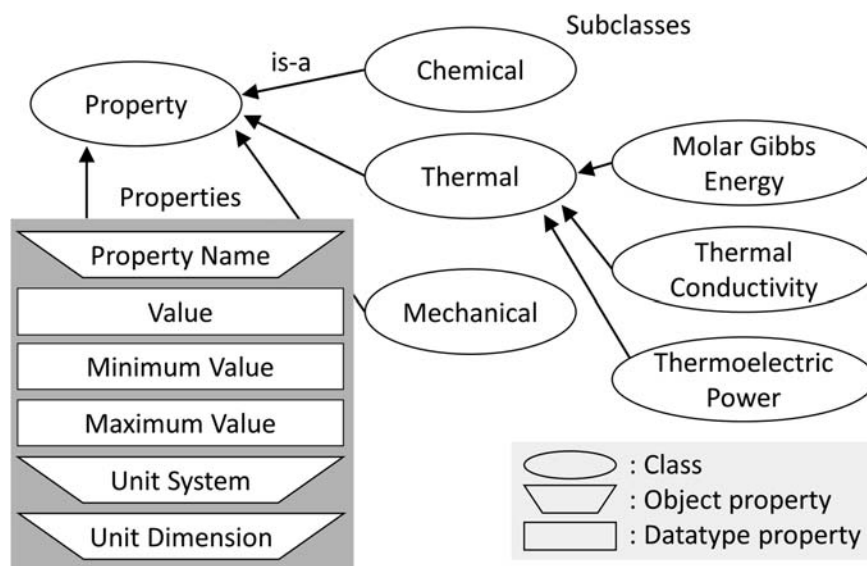
The full mathematical description of materials must be achieved in order to computationally model the biological response they induce. This must include the multiple aspects of materials that define their performance, including both composition and provenance. **Ontologies** are controlled vocabularies that are becoming increasingly important in all “omic” fields. They allow genes, chemicals, materials, etc., to be precisely defined in terms of composition, relationships to other entities, and provenance. Much work in ontology is carried out within the framework of the semantic web, a movement that aims to convert the current World Wide Web (consisting of largely unstructured documents), into a web of data. This will allow computer, instruments, etc., to understand and exchange data easily, without the need for translation between formats and human interpretation of elements of the data, because the information about the data is embedded in the data. This is achieved using ontology languages like OWL (Web Ontology Language) that allow knowledge representation. The data described by an ontology is interpreted as a set of entities (such as a specific type of polymer) and a set of *property assertions* (e.g., “is a homopolymer”) that describe the relationships between the entities. An ontology is a set of **axioms** (premises or starting points for reasoning) that place constraints on sets of entities (or classes) and the types of relationships that are permitted between them. These axioms allow systems to infer additional information from these data without human intervention. Ontologies have been used for a considerable time in biology (e.g., Gene Ontology), but are beginning to be developed for chemistry and materials. An example of chemical ontology is ChEBI. ChEBI consists of ~30,000 terms and acts as a reference for chemical entities, from subatomic particles to polymers, across multiple research domains. Materials-specific ontologies are now being developed. One of the first examples is polymer documentation system of IDC with inclusion of analytical and synthetic concept relations (POLIDCA-SYR), a controlled vocabulary of hierarchically ordered terms (Fig. 9.11). A simple organization of concepts related to materials science might contain four groups of core ontologies: Substance, Process, Property, and Environment. These ontologies give structured definitions of terms, names, and vocabularies for the basic concepts of each core domain (Fig. 9.12). It corresponds to the fundamental data structure of materials property.

9.6.4 Computational modeling of structure–property relationships

When dealing with large numbers of complex materials interacting with biological systems that are not fully characterized, use of physics-based modeling methods such as molecular dynamics simulation or quantum chemical calculations is not feasible. This is because of the extreme time and resource demands of these methods and the need to greatly simplify the materials and environments to make the calculations tractable. **Empirical statistical** and **machine learning methods** (an important subset of **artificial intelligence** methods) are much faster and more flexible than these methods, albeit with a lower degree of interpretability. Computational pattern matching tools like QSPR have been shown to be very useful for materials design and discovery. Although this field of modeling is still relatively new compared to pharmaceutical and related fields, a rapidly increasing number of proof-of-concept studies have demonstrated its capabilities.

**FIGURE 9.11**

The POLIDCASYSR semantic categories. From Adams, N., 2010. *Polymer informatics*. In: Meier, M.a.R., Webster, D.C. (Eds.), *Polymer Libraries*, Springer.

**FIGURE 9.12**

Structure of the property ontology. From Adams, N., 2010. *Polymer informatics*. In: Meier, M.a.R., Webster, D.C. (Eds.), *Polymer Libraries*, Springer.

The key steps in QSPR modeling of complex materials are similar to those required for molecule or drug modeling:

- the relevant molecular, structural, physicochemical, and process parameters of the materials need to be encoded into mathematical *descriptors*, numbers that capture these properties accurately and succinctly
- as there are many ways of encoding these microscopic properties or parameters, methods need to be employed that choose the best subset of descriptors that are most likely to influence a material property, such as cell adhesion, that is being modeled
- the selected subset of mathematical descriptors is then used to train a statistical or machine learning model that is quite often **nonlinear**. Machine learning methods called artificial neural networks (ANNs) are commonly used. They are computational models inspired by the brain that are capable of learning complex relationships and pattern recognition.
- the quality, robustness, predictive power, and domain of applicability (the region of property and descriptor space where the model was generated) of the model must be assessed.
- the model can then be used to screen virtual libraries of materials and identify potential hit materials. Ideally, these experiments would be experimentally verified, and the results could be used to further optimize the model. Analysis of the resultant model can also be used to provide new insight into the underlying material–biological interaction being modeled.

9.6.4.1 Descriptors

As mentioned, one of the most important elements in training a robust and predictive model of materials properties is how the materials structure and other characteristics are encoded mathematically into descriptors. Most machine learning modeling methods generate similar models from the same data set and descriptors, whereas the same modeling algorithm can generate models with large differences in **robustness** and **predictivity** given that same data set encoded by different types of descriptors. As materials are usually more structurally complex than small molecules, and often consist of distributions (e.g., polymer molecular weights, degrees of cross-linking, etc.), encoding these in mathematical descriptors is difficult.

It has been found that polymers can be encoded quite well by using descriptors for monomer molecules or weighted averages of monomer descriptors for mixtures of polymers or block copolymers. This has allowed good, predictive models of many polymer properties to be generated. There is a strong trend to use descriptors that can be mapped back onto molecules to provide clear guidance to chemists as to which functional groups improve or degrade the property under study. The most common form of interpretable descriptors are **molecular fingerprints** such as Morgan fingerprints or signature descriptors. **Kernel methods** in which functions (usually Gaussian) are mapped onto atoms in molecules and used as descriptors have also proven useful. Recent development in deep learning (DL) such as convolutional neural networks (CNNs) have allowed simple graph-based or text string-based representations of molecules and materials to be used, as the CNN generated its own features in the convolutional layers.

9.6.4.2 Feature selection

Just as there are many different ways to describe a person, not all of them useful for a particular purpose, there are a myriad of ways of generating descriptors for materials. As data are often limited for modeling and models with too many independent variables **overfit** the data and predict poorly, it is important to

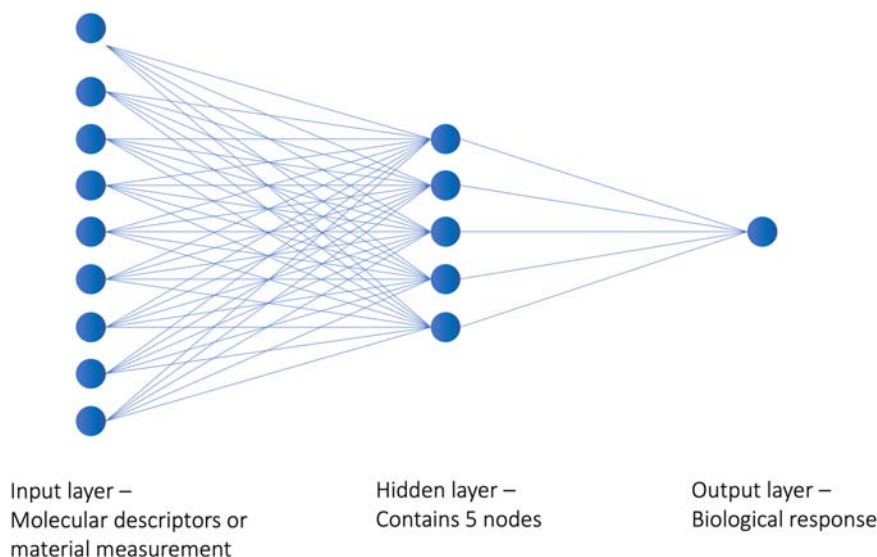
remove descriptors with low relevance to the property being modeled. This is a large and complex topic beyond the scope of this chapter. A couple of common methods of selecting features are discussed here. For statistical regression models, descriptors with the highest correlation with the dependent variable can be successfully added until no further improvement in the model predictions occurs. Conversely, all descriptors can be included (perhaps after eliminating all those that are highly correlated with each other), then removed one-by-one until the model quality starts to decline. More sophisticated methods use sparse feature selection as embodied in the popular least absolute shrinkage and selection operator and multiple linear regression methods. Here all low relevance descriptors are set identically to zero, improving model predictivity and interpretability. This is superior to methods such as PCA because the low relevance descriptors are removed entirely and not simply used to form new latent descriptors by axis rotation. It has been shown that PCA predictions degrade as increasing numbers of low relevance descriptors (essentially noise) are added to the model.

9.6.4.3 Statistical and machine learning models

While quantitative structure–activity relationship (QSAR) was initially a statistical modeling method using linear regression (MLR) and linear logistic regression, the past 3 decades have seen greatly expanded application of diverse machine learning methods, principally, Gaussian Processes (GP), ANNs, and their Bayesian version (BRANN), support vector machines and their Bayesian variant relevance vector machines, decision trees, random forests and their variants such as extreme gradient boost, Naïve Bayes, and k-nearest neighbor clustering methods. More recently, DL algorithms like deep neural networks, CNNs, Generalized Adversarial Networks, associative neural networks, encoder–decoder networks, and recurrent neural networks have exhibited very interesting properties useful for modeling structure–activity relationships. The theory behind these methods is complex and outside the scope of the introductory chapter.

Neural networks are loosely structured on the connectivity of neurons in the brain, albeit with many orders of magnitude lower complexity. Unlike explicitly coded statistical models, where assumptions need to be made about the functional relationships between the independent (e.g., descriptors) and dependent (e.g., biological response) variables, neural networks are universal approximators, capable of modeling any continuous function or relationship given sufficient training data. The same software can be used to model almost infinite number of diverse data sets without recoding. This major advantage, coupled with its ability to objectively fit any linear or nonlinear relationship, has made the neural network one of the most widely used machine learning methods.

The structure of a simple neural network (Fig. 9.13) includes independent variables, such as molecular descriptors encoding materials properties, that impinge on the input layer. Usually all **nodes** (also called neurodes) in the input, **hidden**, and **output layers** are fully connected. The **input data** are distributed to the nodes in the hidden layer via a set of weighted connections in which the weights can be varied. Input to the hidden layer nodes are, therefore, weighted sums of the data from the input nodes. The hidden layer is where the computation occurs. The summed contributions from the input nodes are transformed by so-called **transfer functions** in each hidden layer node. Transfer functions are commonly linear, sigmoidal (e.g., hyperbolic tangent), or more recently a ReLU function (a function in which input below a threshold is set to zero and those above are processed linearly). Again, weighted sums of the output of each hidden layer node are passed to the input of the output node and are

**FIGURE 9.13**

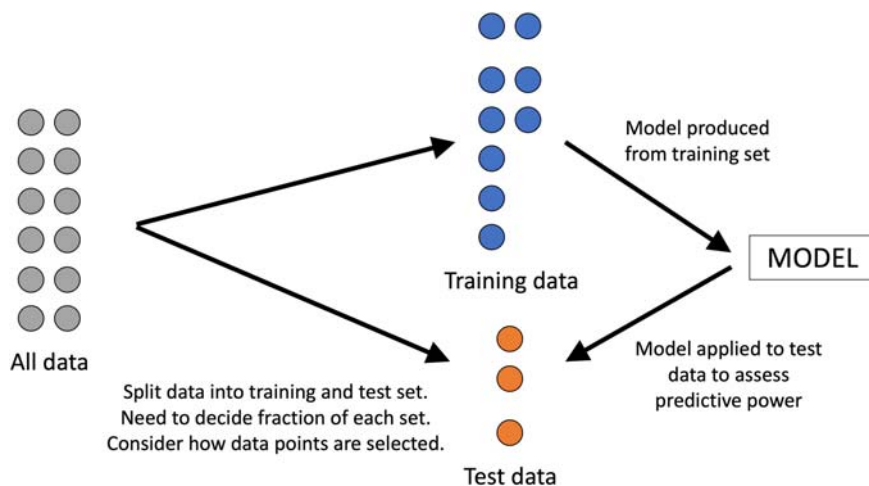
Schematic of a neural network.

transformed by a transfer function in the output node (or nodes as there can be more than one output node). Output transfer functions are normally linear for regression models and sigmoidal for classification models (the sigmoidal function “squashes” the output toward zero or 1).

Initially, the weights of all connections are set randomly. As each training example is presented to the network, the signal is propagated through the network and generates an output. The output is compared to the known output from the training data and the difference (error) is propagated backwards through the network to adjust the weights to minimize the error. This is called backpropagation and is a very common form of neural network. As the training data are repetitively presented to the network, the weights are constantly adjusted until the error is minimal for the entire training data set. Training for too long results in the error of prediction of the training data continuing to reduce, but the ability to predict the properties of new data not used to train the model becomes worse. Thus, neural networks require stopping criteria, the simplest of which is a separate validation set whose properties are predicted by the model. The error in the prediction of the validation set properties initially reduces, reaches a minimum, and then start to increase. The point at which the validation set error is minimal is the point at which the training should stop. There are more complex mathematical methods of training and stopping neural network training such as the use of Bayesian regularization, a topic outside the scope of this chapter.

9.6.4.4 Model validation and assessment of predictivity

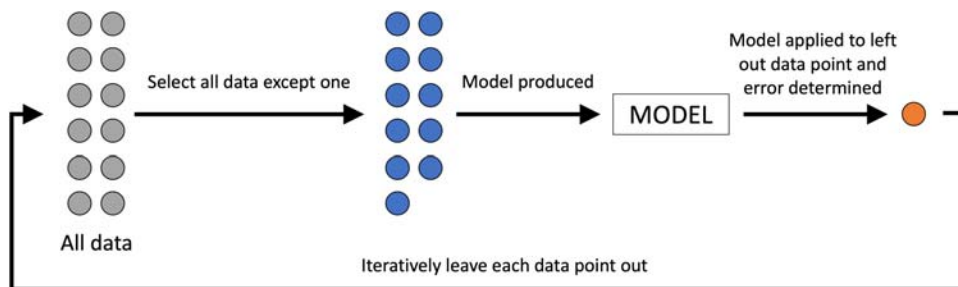
It is very important to validate the reliability and predictive power of machine learning models. This is traditionally done in several ways, each of which has advantages and disadvantages. The ultimate value of a model is its ability to predict properties of data it has not seen and to rationally design materials

**FIGURE 9.14**

Training/test set method to assess model predictivity.

with superior properties. The model could be used to predict the properties of candidates for synthesis to allow their prioritization or to screen a database of virtual materials to identify those with the best properties. These can be subsequently synthesized, and their properties measured and compared with the predictions. This is rarely feasible because of time and resource limitations so an independent test set is most commonly used to assess model predictive power. Here a percentage of the **total data set** is used for a **test set** that is never used in training the model (Fig. 9.14). The properties of the test set are predicted by the model and compared with the known property values for each member. The disadvantages of test sets are that they require removing data that could otherwise be used to train the model, and the method for selecting the tests set and its percentage is rather empirical. The test set is arguably the most accurate method of assessing model prediction power.

Cross-validation is another popular method of assessing model predictivity (Fig. 9.15). Here one or more data points are held aside from the training data and the properties of this or these data predicted

**FIGURE 9.15**

Leave-one-out method to assess model predictivity.

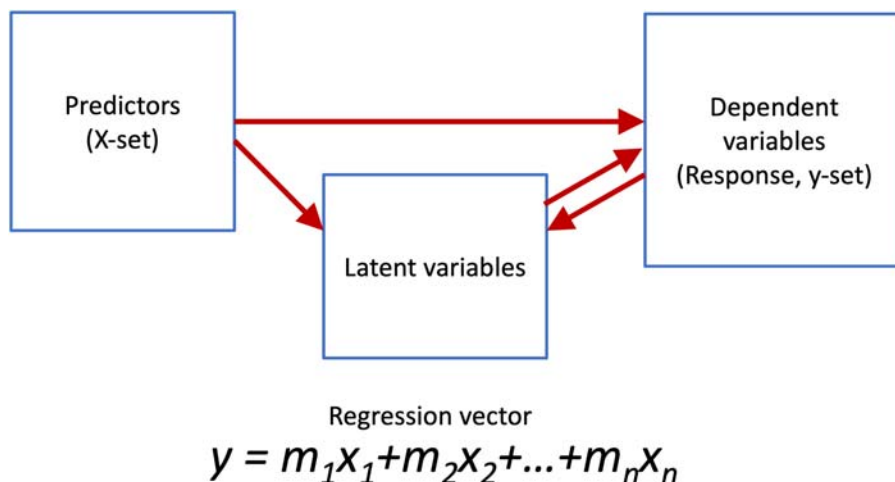
by the model. Each data point, or set of data points, is selected to be held aside in turn and the average prediction error calculated once all data points have been selected for the cross-validation process. If one data point at a time is held back, the process is called leave-one-out (LOO) cross-validation. If n data points at a time are held back, this is called n -fold cross-validation. LOO is particularly useful for small data sets where partitioning part of the data into a test set is not useful. However, because each data point spends most of its time training the model and a small amount of time in the validation set, cross-validation can provide an overly optimistic estimate of model predictivity. Other methods like bagging and boosting involve removing fractions of the data set with or without replacement and using these to assess predictivity.

Regardless of how the model validation is performed, it must be understood that it can only reliably predict properties of materials within or close to its domain of applicability. This is the region of chemistry and descriptor space defined by the training data. While it is possible to make predictions of properties of materials far from the model domain, this usually results in poor predictions as these new compounds contain features that the model has not been trained on.

9.6.5 Illustrative example: correlating measured material properties with cellular attachment

Computational modeling attempts to predict the biological performance of a material from its known composition, molecular, and process properties. Living cells will interact with the surfaces of materials; thus, it is the surface properties of the materials that provide the defining parameter that can be used to describe their biological response (see [Chapter 7 Degradation of biomaterials](#)). It is therefore important to be able to measure various surface properties of materials concurrently with biological measurements, particularly as altering one property (e.g., surface chemistry) may also alter another property (e.g., material stiffness). Where large libraries of materials have been used, it is necessary to have a high-throughput surface analysis methodology that is capable of taking individual measurements from the hundreds to thousands of unique materials. High throughput measurements can be obtained of: a) material surface chemistry (by X-ray photoelectron spectroscopy, time-of-flight secondary ion mass spectrometry (ToF-SIMS), and water contact angle (WCA)); b) mechanical properties (by atomic force microscopy and automated force measurements); and c) biomolecular interactions (by surface plasmon resonance). Once material properties have been measured, the challenge remains to connect them in a meaningful and quantitative way to a biological response. As mentioned, this is difficult due to the complex and multifaceted interactions of cells with materials, whereby cells respond to a material's surface chemistry, hardness, topography, etc ([Fig. 9.4](#)). Simple single correlations with properties that are commonly implicated in cell–material interactions, such as hydrophobicity/hydrophilicity or roughness, typically do not work when considering large and diverse sets of materials.

Multiple linear regression (MLR) approaches, where multiple parameters are mapped to observed biological responses, have been an important step in developing and understanding material structure–property relationships. For example, partial least square regression has been used to correlate univariate properties such as cell numbers, to multivariate materials descriptors such as the hundreds of secondary ions in a ToF-SIMS spectrum ([Fig. 9.16](#)). This method first generates a smaller set of latent variables that describe the main variance in the predictors. The latent variables are then used to generate the MLR. This

**FIGURE 9.16**

Schematic depiction of partial least square (PLS).

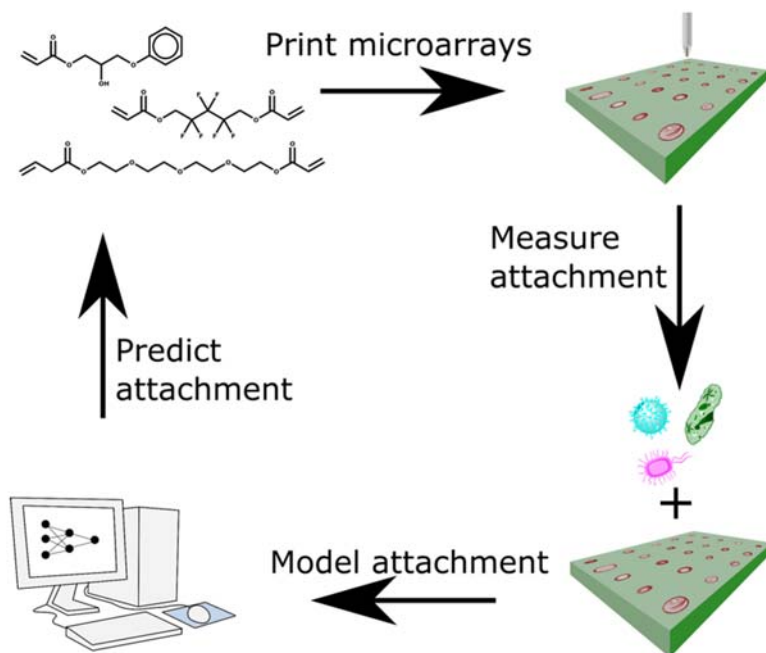
method has been used to link ToF-SIMS spectra with WCA and has also been successfully applied to predict diverse biological responses such as stem cell or bacterial attachment from measurements of surface chemistry. The resultant models definitively demonstrated a link between surface chemistry and biological response.

Ideally, the materials discovery loop (Fig. 9.17) would be implemented, whereby an initial set of observations from a materials microarray are used to construct a model that describes the structure–function relationship. This model would then be used to predict a second generation of materials for a second experimental screen. Each completed loop would improve the model and result in the optimization of the materials composition. The main limitation is the extensive experimental surface analysis required to gather the relevant chemical/physical properties of the materials being studied. That is, the number of materials that can be included is limited to the number of different samples that can feasibly be screened. This also means that the models cannot be used to predict properties of materials not yet synthesized, although they may well be superior to those in the **training set**.

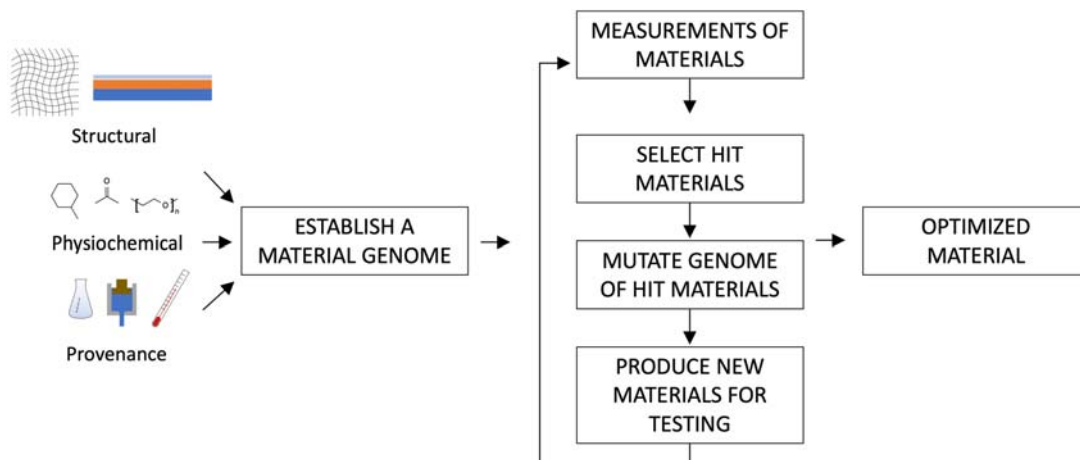
To overcome this constraint, models trained on computed molecular descriptors rather than measured properties are becoming dominant. Materials do not need to be synthesized and analyzed, rather a virtual library of materials can be created where the size of the library is limited by computational power and the materials space to which the model is applicable, rather than experimental resources.

9.6.6 Evolving materials computationally

Analogous to the use of experimental evolutionary methods to discover new materials in regions of materials space far from known areas, computational evolutionary methods can also be used to discover and optimize biomedical materials and materials generally (Fig. 9.18). Genetic algorithms have been shown to explore very large search spaces efficiently and, clearly, this can be done many times faster

**FIGURE 9.17**

The combined experimental and computational materials discovery loop.

**FIGURE 9.18**

The methodology for evolving materials computationally.

in silico than in the laboratory. Briefly, the structural, physiochemical, and synthesis/processing properties of materials are encoded into a string or fingerprint called a materials "genome." An initial small pool of materials is synthesized either randomly or based on prior knowledge. These are tested for

“fitness,” the degree to which their measured properties match those desired, and the best are retained as hits. The genomes of this fittest subset are subjected to *in silico* mutation operations and the materials corresponding to the “mutated” genomes synthesized for testing in the next round of evolution. The mutational operations are usually elitism (where the best materials pass unchanged into the next round), point mutation (where a single element of the genome is altered in some way), and crossover (in which two genomes are split and the fragments joined in new ways). Point mutations explore local regions of materials space, and crossover mutations can jump into new, unexplored areas of materials space. The evolutionary process is continued until materials are obtained with the desired properties, or no further improvement is obtained. An important aspect of computational materials evolution is that experimental data accumulated throughout the process can be used to train ML models of the “fitness landscape” that can be used to minimize the number of new experiments to be conducted.

9.6.7 White box modeling

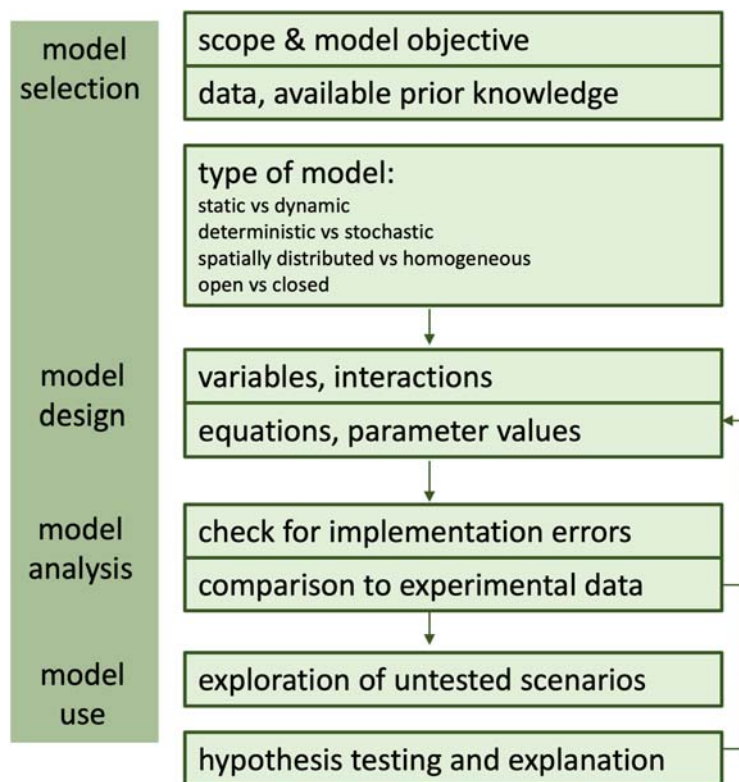
The modeling discussed so far has been focused on **black box models** where the inner workings of the models are complex and difficult to understand and, thus, do not provide ready insight into the structure–function relationship. These modeling approaches are useful when the systems being investigated are complex and poorly understood, or where prediction rather than understanding is the primary goal. An alternative approach is the use of **white box modeling**.

A white box model is defined as “an abstract representation of a complex system in terms of equations or rules and a description of the region (spatially and/or temporally) on which these rules are valid.” The art of establishing a proper white box model consists of creating an appropriate abstract representation with the right level of abstraction suitable for the research question at hand and generating novel insights from it. Importantly, the quality of a white box model is not determined by the complexity of the mathematical equations or the number of variables and their interactions, but by its ability to answer the posed research question and create novel understanding. White box modeling is routinely used in cell signaling, to depict the complexity of the molecular interplay between proteins as signal transduction pathways (see [Chapter 4 Cell signaling](#)).

The following workflow is typically used when establishing a white box model ([Fig. 9.19](#)):

- (1) model selection,
- (2) model design,
- (3) model analysis,
- (4) model use.

First, one must define and simplify the phenomena to be simulated (e.g., cell attachment on bone implants) and make an overview of the data available to calibrate and validate the white box model. This information will enable selecting the proper model type (e.g., model scale, static/dynamic, deterministic/stochastic, spatially distributed/spatially homogeneous, or open/closed). For example, if the amount of cell attachment changes appreciably over time and depends on the particular location on the bone implant, these characteristics might be important to capture, requiring a dynamic, spatially distributed model (e.g., a partial differential equation model).

**FIGURE 9.19**

Flow chart of the generic modeling process. From Voit, E.O., Qi, Z., Miller, G.W., 2008. *Steps of modeling complex biological systems. Pharmacopsychiatry* 41, S78–S84.

Once an appropriate modeling approach has been chosen, the **model components** need to be defined: the variables; parameters; and processes that link the variables. The variables represent the biological and material properties of interest, such as the cells and the mechanical and chemical properties of the bone implant, for example. The processes describe, using mathematical equations, how the variables are linked, e.g., how the material chemistry influences cell attachment. The task of finding these mathematical equations for the cell–biomaterial interface is not trivial. Inspiration can be drawn from fundamental laws and principles in physics, or from the correlations found using the machine learning techniques discussed above. The parameters are the fixed numerical constants necessary to connect the abstract equations of the model to reality. If the model is spatial, an appropriate 1, 2, or 3D geometry must be defined as well.

Typically, one will first formulate the corresponding set of equations and then convert them into a computer code to solve the set of equations numerically. Many software platforms exist for white box modeling, including applications with a graphical-user interface (e.g., COPASI, VCell, CompuCell3D, Comsol MultiPhysics, etc.) or programming languages that allow you to directly program the underlying math (e.g., MatLab, Python, Mathematica, etc.). Before using the white box model, it is important to

test and analyze the model implementation. This step ensures that there are no implementation errors, assesses whether model predictions are consistent with currently available experimental data, and explores the sensitivity of the model predictions with respect to the selected parameter values. If necessary, it might be important to improve the design of the white box model, e.g., include additional variables or change the parameter settings.

Once the mathematical model has been sufficiently tested and calibrated based on particularly designed experiments, the model can be used to answer, “what if?” scenarios, where one might, for example, change the geometry of the device or the chemistry of the material. Importantly, with a white box model, the data can be nondestructively recorded at a higher frequency and spatial resolution than in an experimental setting. The hypotheses derived from the “what if” simulations can be used to design dedicated (high throughput) experiments. The simulation results can even suggest experiments that would not have been apparent before the model. In this way, the white box model can lead to new insights into the mechanisms underlying the biological process at the cell–material interface.

White box models, based on a cellular Potts formalism, have been applied to predict the dynamical forces and cell shapes on micropatterned substrates. The predicted cell contours as well as generated cell traction forces were in agreement with the experimental data. After this validation step, the white box model was used to optimize the migration of cells on ratchet micropatterns. Others have used white box models to design and optimize the release of oxygen or other ions from biomaterials. For example, a COMSOL Multiphysics model was built to predict the oxygen gradients within cell-loaded agarose constructs with varying total cell loads, external oxygen tension, and the presence or absence of the oxygen-generating biomaterial. From these calculations, the dose, geometry, and surface/volume ratio of the oxygen-generating biomaterial could be optimized. Similarly, others have focused on developing a **finite element method** to investigate the local Ca^{2+} ion release from CaP-based scaffolds. Finally, white box models have also been applied to optimize the macroscopic pore shapes of scaffolds to control the kinetics of tissue deposition, e.g., bone formation. It was shown that in cross-shaped pores the initial overall tissue deposition is twice as fast as in square-shaped pores, opening new avenues to improve the speed of bone ingrowth into porous scaffolds.

9.7 Future perspective

The tools for achieving biomaterials discovery are in place. Experimental platforms have now been developed that enable the biological response to a range of materials properties to be assessed in high throughput, including material surface chemistry, topography, and hardness. Coupled with this has been the successful computational modeling of material–biological interactions based upon mathematical descriptors that could easily be applied to virtual polymers. The integration of computational modeling with high throughput materials discovery methodologies will enable the vast chemical space of all possible materials to be successfully navigated to discover new and optimal biomaterials.

An ongoing challenge is ensuring that the scientists working in the varied disciplines required to achieve biomaterials discovery are able to effectively communicate all necessary information. Experimental and computational scientists typically work independently from each other despite the codependence of the two approaches. Other fields have circumvented this problem by establishing data repositories where the results from one research group can easily be accessed by other researchers including all the relevant metadata. Biomaterials discovery would also benefit from improved data sharing and the

establishment of standardized metadata. This is challenging due to the complexities of fully describing materials (including structural, physiochemical, and provenance information) and due to variation in the way that materials are experimentally tested, which prohibits the ability to compare data between laboratories. But the benefit is clear: increased data availability will improve the quality of both black box and white box models that will, in turn, improve the understanding of structure–function relationships. This will provide experimental scientists with the information required to “dial up” the material composition, sample preparation, and product shape necessary for an optimal biomaterial for any biomedical application.

Summary

- Current materials that are used for tissue engineering applications are limited and often have suboptimal interactions with biology.
- Polymer microarrays are a key enabling tool for high throughput materials discovery.
- Synergistic effects are often observed for materials, whereby two components together will produce an effect that would not be predicted from observed the two components solitary.
- The number of potential materials that could be applied to tissue engineering applications is huge (10^{100} different molecules could be synthesized with molecular weights below 1000 Da).
- The most advanced high throughput experimental techniques cannot effectively screen the huge materials space.
- Computational modeling of material–biological interactions offers the potential to screen a larger materials space by producing a model from known interactions and applying the model to a virtual library of materials.
- High throughput material screening data offer the ideal starting point for producing robust computational models.
- Bacterial attachment and stem cell attachment to materials has been successfully modeled using only mathematical descriptors derived from monomer/polymer structure.
- Rapid and effective identification of optimal materials for a given tissue engineering application will be achieved when high throughput materials discovery methodologies are used in tandem with computational modeling.

Classic experiment

One of the first studies that assessed the biological properties of a library of polymers in order to establish a structure–property relationship was conducted by Brocchini et al., in 1998. To achieve this initially a library of 112 unique polymers was formed by copolymerizing 14 different diphenols (the A component) with 8 different diacids (the B component) in all possible combinations to form alternating (polyAB) polymers. These were not assessed on an array, but rather as coatings on glass coverslips. The large number of materials included within this study enabled the relationship between the polymers’

composition and properties such as the WCA and glass transition temperature (T_g) to be explored. For assessing the biological response to the materials, a subset of 42 copolymers were selected and produced as coatings that were then exposed to fibroblast cells that attached and proliferated on the materials. The proliferation of cells was compared with the WCA of materials, and although proliferation was highest at the lowest WCA, this study highlighted the complex nature of cell interactions with materials—by changing the backbone of the polymer to include oxygen cell proliferation was increased despite

Classic experiment—continued

the WCA being the same. This suggests that cells respond to specific chemistries in addition to general surface properties such as surface energy, and that the hydrophilicity/hydrophobicity alone is not sufficient to describe the cell response.

Data for polymers containing glutaric versus diglycolic acid in their backbones is shown [Fig. 9.20]. For each polymer series, identical linear pendent chains (methyl, ethyl, butyl, hexyl, octyl, and dodecyl)

were evaluated. For the nonoxygen containing glutaric acid cell proliferation decreased strongly in a linear fashion as the contact angle increased. Replacing single methylene groups by oxygen atoms in the backbones of the glutarate series dramatically changed the cellular response. In the polymers derived from diglycolic acid cell proliferation was supported uniformly even though the contact angles for some of these polymers exceeded 90 degrees.

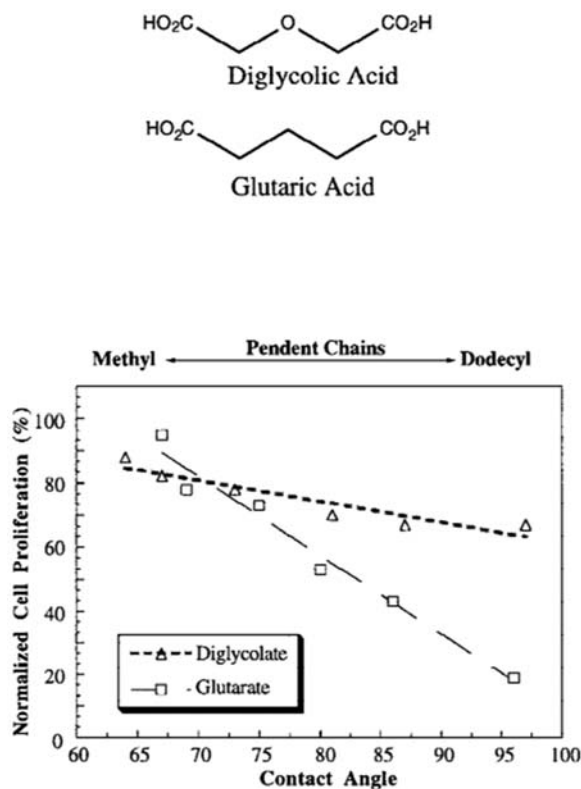


FIGURE 9.20

Comparing the proliferation of fibroblasts with WCA—toward establishing a structure—function relationship. From Brocchini *et al.*, 1998. *Structure-property correlations in a combinatorial library of degradable biomaterials*. *J Biomed Mater Res* 42, 66–75.

State-of-the-art experiment

Infection by pathogenic bacteria on implanted and indwelling medical devices is a major cause of device failure and hospital acquired infection with significant costs to hospitals and increased patient morbidity and mortality. Attempts to minimize this important medical issue have included the development of materials with inherently low bacterial attachment but have been limited by the poor understanding of how bacteria interact with material surfaces. However, application of a high throughput materials discovery strategy enabled a new class of materials that are resistant to bacterial attachment to be discovered. The large number of material–biological interactions conducted in these experiments provided new insight into the way bacteria, specifically *Pseudomonas aeruginosa* (PA), interacted with polymer surfaces. In particular, the sur-

face properties associated with preventing bacterial biofilm formation were identified. The hydrophilicity and molecular rigidity (as a composite parameter α) of the hydrocarbon pendant groups of a library of polyacrylates was observed to correlate with bacterial attachment. This simple relationship was used to computationally screen possible hit materials to predict hit compositions for experimental testing. Using this approach, a novel polymer, poly (cyclododecyl methacrylate) (pCyDMA), resistant to bacterial biofilm formation was identified. Coatings of pCyDMA reduced biofilm formation of PA, *Proteus mirabilis*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus* by 32–300-fold (average 55-fold) compared with an uncoated silicone catheter and outperformed all materials present in initial screens.

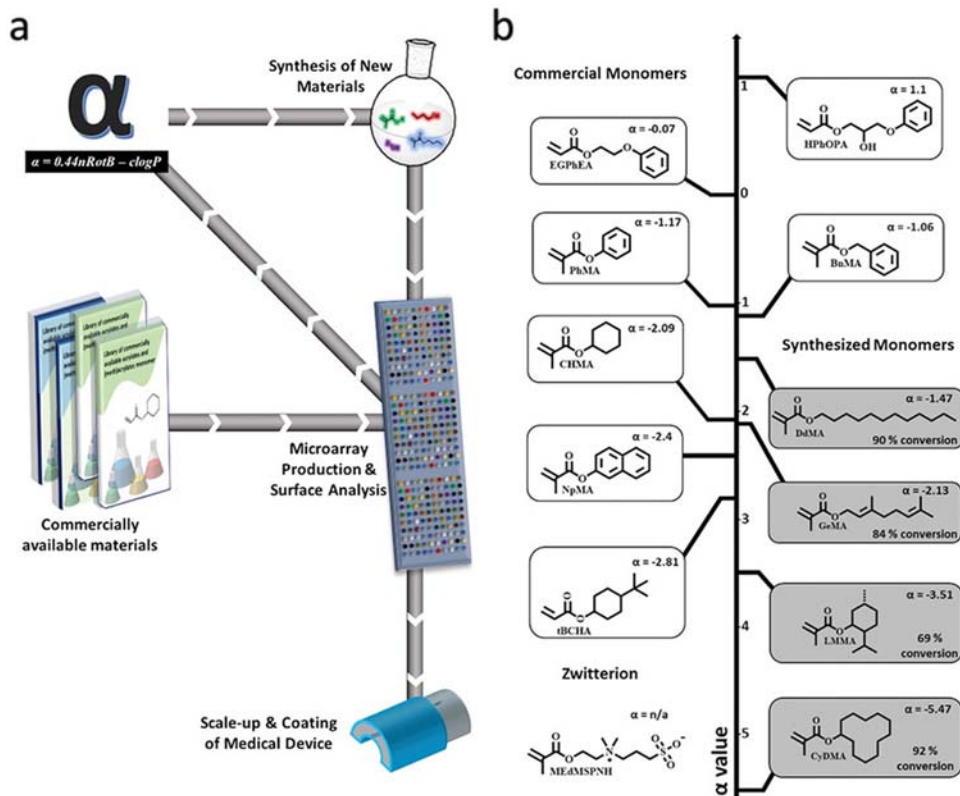


FIGURE 9.21

a) Process for developing and validating a predictive model. Data from hundreds of commercially available materials together with material properties are used to generate models that predict new untested materials which are synthesized and tested. This repeated cycle refines the theoretical model. (b) Materials library screened including 12 monomers with different α values. From Dundas et al., 2019. Validating a predictive structure-property relationship by discovery of novel polymers which reduce bacterial biofilm formation. *Adv Mater* 1903513.

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9.9 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
 1. What is the difference between hypothesis led and high throughput materials discovery?
 2. What experimental circumstances are best suited to high throughput screening approaches?
 3. What are the key requirements of a supporting substrate and its coating for high throughput materials discovery platforms?
 4. Which material properties need to be considered when developing a new biomaterial?
 5. What key consideration must be made when developing a library of materials where a single property is intended to be varied systematically?
 6. What is synergy in a materials discovery context and why is it important to consider?
 7. How many experiments would be necessary if assessing five different experimental parameters using a Design of Experiments approach?

8. What makes a biological assay compatible with a high throughput screening experiment?
 9. What are the four main steps in generating a QSPR model?
 10. What are the most important factors that determine the robustness and predictivity of QSPR models?
 11. Why is feature selection so important in QSPR models?
 12. How are QSPR models assessed for their ability to predict new data?
 13. What determines how reliably strong QSPR models can predict the properties of very large libraries of virtual materials?
 14. What are the disadvantages of using experimentally determined descriptors, such as ToF-SIMS ion peaks, as descriptors in QSPR models?
 15. Why are evolutionary methods needed in biomaterials discovery?
 16. What is the difference between a black box and a white box model?
 17. Give an example of a black box model and a white box model.
 18. What are the three main model components of a white box model?
 19. What are the advantages of white box modeling for biomaterials discovery?
 20. Which checks are typically done to check the model implementation before starting to simulate “what if” scenarios?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:
1. What are the biggest obstacles to the rationale design of biomaterials?
 2. Describe the different approaches to developing a materials library and their advantages and disadvantages for biomaterials discovery.
 3. Why is it possible to reduce the number of experiments required by applying a Design of Experiments approach?
 4. Think about a system with four experimental parameters, which combinations of experimental conditions should be selected in order to create a balanced design for a Design of Experiments approach using a partial factorial design with $P = 1$?
 5. Using Fig. 9.10, describe what different biological assays might be used at different stages of a materials discovery project.
 6. Why are computational methods useful in biomaterials discovery?
 7. Describe why machine learning models are more efficient than hard coded rules-based models.
 8. Considering a library of materials, for example, a collection of 100 different polyacrylates that each had different chemical pendant groups, design an experiment to apply evolutionary methods to biomaterials discovery.
 9. Give two examples of white box models and their findings in the field of biomaterials discovery.
 10. If given unlimited resources, describe what the ultimate biomaterials discovery project would look like.

Challenge-based learning

Establishing a machine learning model for peptide-induced cardiomyocyte maturation

Note for teachers: A challenge-based learning (CBL) user guide can be found at

www.jandeboerlab.com/TissueEngi

neering with instructions and tips to run an effective CBL teaching session.

Background and vision

Biomaterials are widely used as scaffolds in tissue engineering and to produce medical implants. The response to implanted biomaterials by the body is often not optimal and can lead to infection, encapsulation, and can result in poor differentiation of stem cells that attach to them. It is well documented that the physicochemical properties of biomaterials determine the cellular response and their modifications can optimize cell–material interaction. Combinatorial chemistry allows us to produce hundreds to thousands of different polymers and high throughput screening can identify the most appropriate hit for a particular biological or biomedical applications. Furthermore, high throughput screening can be used to generate QSAR models which are key for further biomaterial optimization. The long-term vision of this research is that in-depth knowledge of material properties and cellular response to many different materials will lead the way in bioactive material discovery.

Motivation and stakeholders

Induced pluripotent stem cells (iPSCs) can be differentiated into cardiomyocytes and are seen as potential source for the generation of cardiac patches to repair tissue after an infarct. However, cardiomyocyte differentiation is not yet fully achieved, with current protocols showing that the cells are still in an immature stage. Polymers are used to grow patches of iPSC-derived cardiomyocytes and this field can be boosted if biomaterial engineers are able to model the relationship between polymer properties and cardiomyocyte maturation. Solutions to mitigate this problem should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as cell biologists, material scientists, computational biologists, bioengineers, and surgeons (orthopedic, plastic/reconstructive, and cardiothoracic).

Problem definition

Polymer backbones can be modified with peptides in order to functionalize them with an application in mind. Peptides are

chemically very diverse and will lead to a wide diversity of biological activity. So far, no libraries of peptide-modified polymers exist, the polymer material properties have not yet been defined, and the cell response still needs to be monitored. Thus, using cardiomyocyte maturation as an example, there is a need to create an HTS platform to model and study biomaterial libraries and its impact on cell–material interactions.

Challenge

To design an HTS platform to analyze cellular and physicochemical properties of a polymer to which a library of peptides is added, arrayed, and in which cell response and material properties can be measured and modeled.

Learning framework

Reading the Biomaterial discovery chapter and related literature will help you to understand the following:

1. The ways to prepare polymer libraries.
2. The methods and techniques to measure biomaterial properties.
3. The ways to measure biological readouts in polymer libraries.
4. The modeling methods to describe the quantitative structure–activity relationship (QSAR) of materials to cell response.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

5. The methods to produce peptides and how they can be attached to a polymer backbone.
6. The types of polymers used so far to optimize cardiomyocyte maturation.
7. The cardiomyocyte differentiation stages and which markers are used to identify them.
8. The ways Design of Experiments (DoE) can assist scientists and bioengineers in designing experiments.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

9.10 Glossary

Artificial intelligence is the ability of a machine to perform a task that would usually require the natural intelligence displayed by humans or animals.

Axiom is a statement that is taken to be true, to serve as a premise or starting point for further reasoning and arguments.

Bioactivity is an effect on, interaction with, or response from a living tissue or cell.

Biocompatibility is the ability of a material to perform its function with an appropriate host response without causing any undesirable local and systemic effects.

Biodegradation is the mechanical, physical, and chemical modification of a material by a biological environment and associated cells to break it down to its monomeric form.

Biological assay is an analytical method to determine the concentration or potency of a substance by its effect on living animals or plants (in vivo), or on living cells or tissues (in vitro).

Biological environments are cellular and physical surroundings of an organism, include the factors that have an influence in their survival, development, and evolution.

Biological molecule is a term for molecules present in organisms that are essential for one or more biological processes, such as cell division, morphogenesis, or development.

Black box model is a system which can be viewed in terms of its inputs and outputs (or transfer characteristics), without any knowledge of its internal workings.

Cell phenotype is the set of observable characteristics or traits of a cell.

Chemical design space is the property space spanned by all possible molecules and chemical compounds adhering to a given set of construction principles and boundary conditions.

Chemical diversity is the variety of chemical compounds present in an environment.

Chemical resolution is the degree of detail that can be achieved in measuring differences between molecules in an environment.

Cross-validation is a model validation technique for assessing how the results of a statistical analysis will generalize to an independent data set.

Descriptors are words or features that serve to describe or identify something.

Design of Experiments is an organized method to study the relationship between multiple input variables and key output variables.

Empirical statistical (method) is used to describe and summarize empirical data in a convenient way.

False negative is an error where the test result incorrectly indicates the absence of a condition when it is present.

Example An individual is diagnosed negative for diabetes when they have the disease.

False positive is an error where the test result incorrectly indicates the presence of a condition (such as a disease when the disease is not present).

Finite element method is a widely used method for numerically solving differential equations arising in engineering and mathematical modeling.

Full factorial design is an experiment design where all the levels of all the factors are combined with one another. For example, if there are two factors, and each factor has three levels, then the possible combination to perform the experiment is $2^3 = 8$.

Genetic methods (materials design) are iterative approaches to materials design where materials are repeatedly synthesized, tested, and then slightly modified in order to optimize the structure of a material for a particular performance criterion.

Hidden layer is a layer in between input and output layers in a neural network, where artificial neurons take in a set of weighted inputs and produce an output through an activation function.

Homopolymer is a chain of chemically linked molecules that are identical to each other.

In vitro means studies performed with microorganisms, cells, or biological molecules outside their normal biological context.

In vivo means studies in which the effects of various biological entities are tested on whole, living organisms, or cells, usually animals, including humans and plants.

Input data is the first set of data that a neural network uses to make a prediction.

Kernel methods are a class of algorithms for pattern analysis with the general task to find and study general types of relations (for example, clusters, rankings, principal components, correlations, classifications) in datasets.

Locally optimal properties are conditions where small increases or decreases to a material's properties do not improve the material's performance even though the conditions do not achieve the optimal performance.

Machine learning methods are approaches where a computer is able to train itself to perform a task, including supervised/unsupervised learning and reinforcement learning.

Material stiffness is the measure of a material's ability to resist deformation when acted on by an external force.

Materials design space refers to all the possible different material formulations.

Mechanical interaction is the capacity of an object to influence the motion of another object.

Model components are the variables associated with the subject of a model and the processes that link the variables together.

Molecular fingerprinting is a method of encoding the structure of a molecule in which a series of binary digits (bits) represents the presence or absence of particular substructures in the molecule.

Nodes are computational units that have one or more input connections. Input from the data is combined with a set of coefficients that either increase or diminish it, thereby assigning significance to inputs according to the task the algorithm is trying to learn.

Nonfouling refers to the ability to resist the adsorption of biomolecules, such as proteins, or adhesion of cells.

Nonlinear is a system in which the change of the output is not linearly proportional to the change of the input.

Ontologies are detailed formalizations of a certain area of knowledge using a conceptual scheme. It consists of a data structure containing all relevant classes of objects and rules (theorems, restrictions) adopted in this area.

Output layers are the last layers that produce the final computational results in neural networks.

Overfit the production of an analysis that corresponds too closely or exactly to a particular set of data, and may therefore fail to fit additional data or predict future observations reliably

Partial factorial design consists of a carefully chosen subset of the experimental runs of a full factorial design.

Physicochemical involves both physical and chemical properties of a substance.

Predictivity is a condition when it is possible to accurately forecast future states of the system.

Provenance is a detailed record of model organism origin and history, to preserve its scientific significance.

Quantitative structure–property relationships are relationships between chemical structure and biological activity or chemical property of a biomaterial.

Readout is a visual record of the experiment.

Regenerative medicine deals with the process of replacing, engineering, or regenerating human or animal cells, tissues, or organs to restore or establish normal function.

Robustness is the property of a model when its outputs and forecasts are consistently accurate.

Self-oxidation is the property of a substance to react with itself in the presence of oxygen.

Structure–function relationship is the reliance relationship between the physical and chemical properties of a material on its functional properties.

Synergistic effects are interactions or cooperation giving rise to a whole that is greater than the simple sum of its parts.

Synthetic polymers are human-made substances or materials consisting of very large molecules and composed of repeating units.

Test set is a data set used to provide an unbiased evaluation of a final model fit on the training data set. The test set is not used to create the model.

Tissue engineering is the design and fabrication of living replacement devices for surgical reconstruction and transplantation.

Topography is the architectural landscape of the surface of a (bio)material.

Total data set is a collection of analytical results for all required variables.

Training set is the data initially used to create a computational model.

Transfer function is the ratio of the output of a system to the input of a system.

White box modeling is an abstract representation of a complex system in terms of equations or rules and a description of the region (spatially and/or temporally) on which these rules are valid.

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Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

Microfabrication technology in tissue engineering

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10.1 Learning objectives

After reading this chapter you will be able to:

- Learn the history of microfabrication and its impact on science, technology, and society.
- Recognize the advantages of microfabrication technology over macroscale approaches.
- Be able to identify major microfabrication techniques used in tissue engineering (TE).
- Understand the concept and working principle of each microfabrication process and the strategies used to assemble microtissues into larger, hierarchical constructs.
- Be able to describe how each microfabrication technique is utilized in various TE applications.

There's plenty of room at the bottom.

Richard P. Feynman, 1959.

I don't know how you were diverted

You were perverted too

I don't know how you were inverted

No one alerted you

George Harrison, 1968.

10.2 Introduction

10.2.1 Background

In the last half century, the advent and development of **microfabrication** technologies have contributed tremendously to advancing many facets of science and engineering. Microfabrication, by definition, is a process by which a structure with micrometer dimensions and resolution is fabricated. A micrometer in turn is one thousandth of a millimeter. Per definition, the micro(meter) scale ranges from 100 μm , which is about the diameter of a human hair, to 0.1 μm , which is about half of the diameter of a hair-like "cilia" on the surface of a lung epithelial cell. Often, a microfabrication process consists of several pertinent techniques in order to create the final desired structure. Developing structures on

the micrometer scale clearly offers several advantages over macroscale structures. First, miniaturization can substantially decrease the cost per run of an experiment by reducing the amount of materials needed for structure development and of samples, reagents, etc., during conduction of an analysis or synthesis. Second, when fully implemented, the microfabrication process can be achieved using automated systems, which results in significant increase in productivity. Third, miniaturization also makes it possible to integrate numerous structures or devices in a confined space, therefore enabling efficient high-throughput experiments.

In a historical perspective, microfabrication was conceptualized and came into fruition in the realm of electronics; the fabrication of integrated circuits involves a series of microscale techniques, such as thin film deposition, lithography, etching, and doping on silicon substrates in semiconductor manufacturing. Needless to say, electronics has had and will continue to have a vital and revolutionary impact on our society, completely transforming our everyday lives. More recently, the same techniques are being used to fabricate **Micro-Electro-Mechanical Systems (MEMS)** in the form of respective sensors and actuators, which have already been commercialized and widely used. For example, pressure transducers are used to monitor tire pressure in automobiles. Inkjet printers utilize piezoelectric actuators that undergo vibrating motion upon applied electricity, resulting in a continuous formation of ink droplets. Digital projectors have an array of micromirrors that are electrostatically actuated to flip and reflect light pixels of digital images.

Microfluidics has emerged as one of the latest innovative microtechnologies that allows high-throughput and precision analytics. Microfluidics is a technology that controls the flow of fluids in amounts ranging from tens to hundreds of microliters. Therefore, manipulating various fluid flows within a microfluidic device allows for sensitive and accurate separation and detection of analytes, using only small amounts of sample reagents. The microfluidic concept is also combined with MEMS-based analytical devices, termed **Micro Total Analysis Systems (μ TAS)**, with the goal of fully automating the analytical process on an integrated microchip. An extension of such an approach is also referred to as **lab-on-a-chip**, which generally integrates and automates multiple laboratory procedures into a system that fits on a chip of a few square centimeters in size.

Microfabricated devices have been traditionally made using rather hard and stiff materials, such as silicon, glass, and metal. However, **polydimethylsiloxane (PDMS)**-based elastomers have become a popular material of choice in recent years for several reasons. First, PDMS elastomers are mechanically stable and yet not brittle, as they possess elasticity, which, among others, allows the realization of **microactuators** based on deflectable or stretchable membranes, such as microvalves or cell stretchers, respectively, and facilitates the demolding of the micromolds. Second, they are easily fabricated by a simple **mix-pour-cure-peel sequence**. Third, they are transparent, which makes it possible to visualize and observe the experimental process with an optical microscope. Fourth, they are permeable to gas, chemically inert, and biocompatible for cell culture, therefore highly applicable for biological experiments. Fifth, the PDMS structures can be easily bonded with a substrate (i.e., glass, etc.) to achieve microchannels and –chambers/-reservoirs.

10.2.2 Microfabrication meets tissue engineering

Microfabrication consists of the design, creation/production, and application of patterns/structures, devices, and systems at the micrometer scale. The properties of materials at this length scale are

significantly different from those at a macroscopic scale, and for this purpose, microfabrication has been receiving much attention in recent years. Developing materials used in biology and medicine can benefit greatly from the evolution of microfabrication, as it can optimize the interactions occurring at the interface between materials and biological systems with micrometer-scale precision. To put this in perspective: a fibroblast is about 50–100 μm long, its nucleus about 6 microns in diameter, and focal adhesion complexes are between 0.25 and 10 μm large. By providing the possibility to tailor detailed (surface) topography as well as overall structure, these microfabrication technologies allow for the development of new materials possessing unique properties.

In the field of tissue engineering (TE), many strategies involve the use of various biomaterials as **artificial extracellular matrices** (ECMs) to engineer tissues or organs, in order to provide suitable **micro-environments** for cells or tissues to function properly.¹ There is a substantial need to engineer these artificial tissue constructs on a micrometer scale, in order to manipulate the architecture to control cellular behavior and to pattern the inner structure (see [Chapter 8 Cell–material interaction](#)).² In addition, micrometer-sized biomaterials could be useful as injectable delivery vehicles of therapeutic molecules in clinical applications (see [Chapter 12 Controlled release strategies in tissue engineering](#)). Naturally, several microfabrication techniques that are currently available in electronics, MEMS, and microfluidics are increasingly adopted to engineer materials used in TE to control their various properties: size, overall shape, spacing, architectural details, porosity, surface texture, and chemistry.^{3,4}

In this chapter, we will examine the concept, working principle, and advantages of microfabrication technologies that are actively employed in various TE applications, including photolithography, soft lithography, and microfluidics. In addition, strategies to assemble microtissues into hierarchical constructs will also be discussed.

10.3 Microfabrication techniques in tissue engineering

10.3.1 Photolithography

The term “**photolithography**” is created by adding the prefix “photo”, the Greek root meaning “light”, to the word “lithography”. Lithography is a printing method that utilizes prepatterned stones (i.e., “litho”) or metal plates to transfer patterns onto desirable media, such as paper, in a high-volume fashion. In the context of microfabrication, photolithography refers to the set of techniques that features the use of light to generate microscale patterns and to efficiently transfer the patterns in or onto the desired substrates. Photolithography is the core technology in fabricating microchips in the semiconductor industry. Using photolithography, huge amounts of miniaturized electrical components, such as transistors, diodes, resistors, and capacitors, can be fabricated altogether into a single chip, with sophisticated electrical wiring that jumps across each other three dimensionally to interconnect these electrical components. For example, the numbers of transistors (representative building components for semiconductor-based electrical circuits) that can be packed into a single central processing unit have reached the order of billions already in the 2010s.

Despite its amazing fabrication capability, photolithography was majorly developed to work with materials such as **silicon**, metals, and **photo-reactive resins** that are less relevant to the needs of TE. Historically, the adaptation of photolithographic techniques to TE marks an important initial effort to pursue the high-resolution patterning of cells and biomaterials, which have led to the development

of photoreactive biomaterials and stereolithography-based bioprinters. In this subsection, we will briefly introduce the principle and workflow of photolithography. Then, using photolithography as an example, we encourage the readers to think together with us about how a new fabrication technique can be adapted to TE purposes.

In essence, photolithography involves the fabrication of a thin (from a few nanometers to hundreds of micrometers) film with desired micropatterns on top of a substrate (usually a silicon wafer). The process starts with the application of solutions containing photosensitive polymers, called “photoresists” (PRs), onto a substrate to form a uniformly thin liquid film. Then, the liquid film is solidified by heating up the substrate from beneath to drive the solvents out from the liquid film. Next, as shown in Fig. 10.1, a mask is prepared with patterned chrome on a glass plate. Then, a thin PR film, prepared

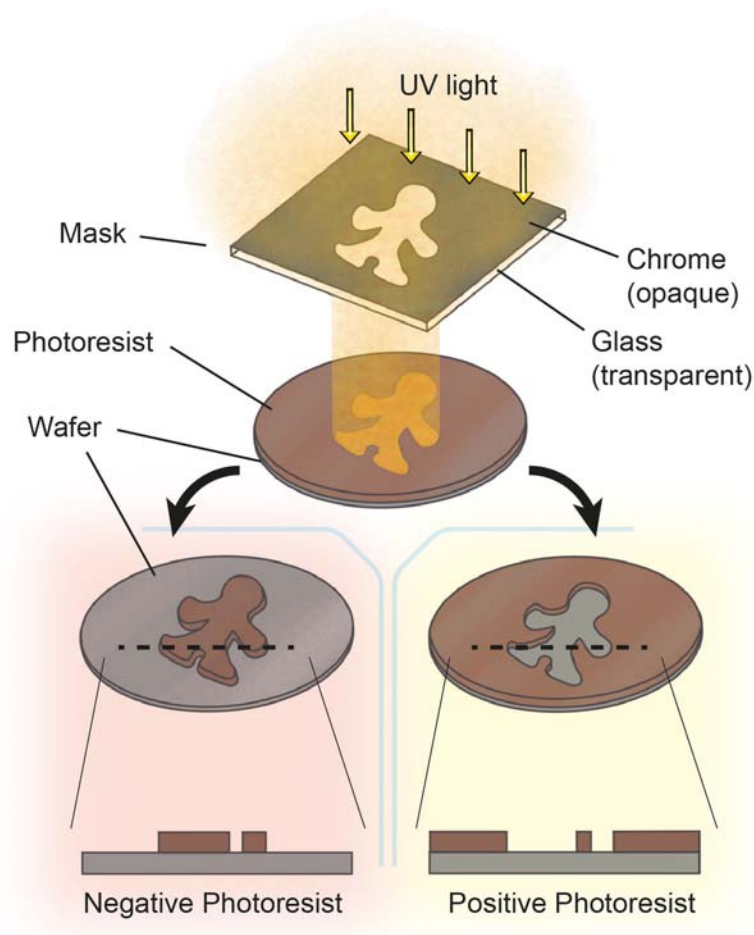


FIGURE 10.1

The typical workflow of photolithography to achieve the fabrication of microscale structures.

on the surface of a silicon wafer, is brought in proximity to the mask. Next, by shining ultraviolet (UV) light down toward the **photomask**, the thin PR film is selectively exposed to light to trigger localized chemical reactions so that the PR can then be selectively dissolved to achieve the desired patterns. Depending on the PR type, the achieved microstructure can be either identical to the light pattern or the opposite: for negative-tone PR, which are designed to remain undissolved after exposure to UV light and development, the fabricated pattern shall be identical to the light pattern; for positive-tone, which are designed to be dissolved after exposure, the fabricated pattern shall be opposite to the light pattern.

As shown in Fig. 10.2, there are various scenarios for the application of photolithography to TE, and the main three types can be categorized based on how cells interact with the fabricated microstructures: cells-on-the-microstructures, cells-in-the-microstructures, and cells-surrounded-by-the-microstructures.

First, cells can be directly inoculated on top of the microstructures (i.e., “cells-on-the-microstructures”) and the microstructures can act as tissue scaffolds with microscale features serving as geometrical cues to guide cell behavior. In order to culture cells on the microstructures, the materials have to be nontoxic to cells and promote cell adhesion, which explains why PRs are not directly used in most applications; the organic solvents and photoinitiators in the PRs are generally toxic and the surface of the PR structures do not have favorable surface characteristics for cell adhesion. Instead of directly seeding cells on top of the micropatterned thin PR film, it can serve as an **etching/deposition mask** or master mold to transfer the patterns onto other materials that can promote cell interactions and activities. In the first case, the PR is used to locally protect the silicon substrate from silicon etchants (i.e., chemicals that specialize in the removal of silicon) and therefore achieve the patterning of silicon. Such patterned silicon has been further coated with silicon oxide and used for cell biology research. For example, epithelial cells were found to elongate and align along patterns in the form of grooves and ridges from silicon oxide with

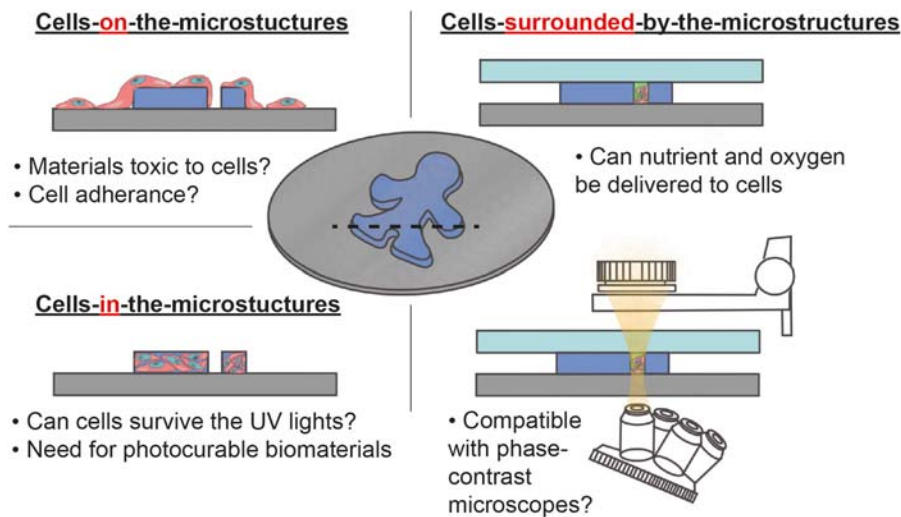


FIGURE 10.2

Various scenarios for the application of photolithography to TE. First, cells can be directly inoculated on top of the microstructures. Second, cells can become part of the microstructures. Third, cells can be confined in the microscale chambers/reservoirs or channels.

feature dimensions of 70 nm.⁵ Such phenomena have been utilized to engineer cell alignment in 3D tissues⁶ (see 10.5 Classical experiment). More background on micropatterned control of cell shape is described in Chapter 8 Cell–material interaction.

Second, cells can become part of the microstructures (i.e., “cells-in-the-microstructures”) and form into thin tissues with microscale geometries. Though the microstructures are thin compared to millimeter-sized tissue, the thickness of several hundreds of micrometers is already multiple times thicker than a monolayer of cells in traditional tissue culture dishes (which is often referred to as “2D cell culture”) and is considered an effective method for three-dimensional (3D) cell culture. For the cells to dwell inside, the PRs used in traditional photolithography have to be replaced with photo-cross-linkable hydrogels mimicking the natural ECM of the *in vivo* tissue. In addition, the light source must be able to adequately initiate the chemical reaction of the precursor solution so that the gelation occurs efficiently while not damaging the encapsulated cells. The most commonly used light source is UV, as UV-activated initiators are often used to start the polymerization process of vinyl-based molecules. Various kinds of both natural and synthetic polymers (such as poly (ethylene glycol), gelatin, etc.) have been rendered photo-cross-linkable by employing chemical modification schemes to conjugate vinyl groups, which undergo radical polymerization triggered by photo-reactive initiators.

Third, cells can be confined in microscale chambers/reservoirs or channels (i.e., “cells-surrounded-by-the-microstructures”) and be subjected to various spatially defined stimuli, such as temperature or chemical gradients. To culture the cells within the confined microenvironment, the first issue is the nutrient and oxygen delivery to the cells through the surrounding materials. Semipermeable materials or structures that can allow the penetration of gas as well as macromolecules, such as porous membranes above or below the microtissues⁷ or lateral rows of pillars with narrow spacing,⁸ are often preferred for such purposes. In addition, it is also desirable that the materials are transparent so that optical imaging methods (such as phase-contrast microscopy) can be used for the investigation of the engineered tissue. Nowadays, these ideas have led to the concept of organ-on-a-chip or human-on-a-chip, which aims to build models of human tissues in a minimalist fashion for the ease of inspection and analysis. The details of these fields are covered in Chapter 19 Tissue engineering of organ systems.

10.3.2 Soft lithography

The root “litho” (meaning stone) in the word “lithography” not only memorizes the historical importance of limestone plates from which the patterns were transferred to paper, it also conveys a “solid” and “expensive” image of the lithographic methods that need hard and costly materials and equipment, respectively. Modern facilities for photolithography require specially designed **cleanrooms** to tightly control temperature and humidity, maintain extremely low levels of dust or airborne particles, and are shielded from light that would unintentionally expose PR. From the perspective of bioengineers, photolithography is expensive, hard to access, and restricted to solid and, in best case, bioinert materials. Such limitations led to the conception of **soft lithography**, which is a set of techniques that evolves on the use of a patterned soft elastomer as the mask, stamp, or mold to fabricate (bio)materials with minimized reliance on large and expensive photolithography facilities. In TE, soft-lithographic

techniques are used to control the size, shape, and pattern of micrometer-scale tissue constructs, among which microcontact printing (μ CP) and tissue micromolding are the most representative ones.

μ CP is essentially a stamp printing technique in which a microfabricated stamp is inked with a bioactive molecule (Fig. 10.3). The coated stamp is then pressed against a surface, resulting in a transfer of the coated material from the stamp to the substrate. μ CP has quickly become a popular technique for patterning substrates for its obvious simplicity and cost-effectiveness, and has been used in TE applications, such as the control of cell adhesion by printing patterns of various cell-adhesive molecules on biomaterial substrates. For example, adsorption of ECM proteins and subsequent cellular adhesion and organization on a variety of substrates (e.g., glass, silicone rubber, polystyrene) could be effectively controlled by patterning the surface wettability, and the patterning of cell-adhesive peptides (e.g., Arg-Gly-Asp (RGD) and Lys-Arg-Ser-Arg (KRSR)) has been achieved using μ CP to control cell behavior on substrates.

In **tissue micromolding**, liquid precursors of **biopolymers** are poured onto a flat substrate and capped subsequently using the soft mold derived from a PR master with hollowed-out geometries so that later the solidified biopolymers can adopt the shape of the hollows in the mold (Fig. 10.4). The soft, elastomeric molds are especially useful compared to molds made of solid materials, such as silicon and metals. A soft mold can fully conform to a flat substrate (such as from microscopy slide or coverslip glass) and also a curved one (Fig. 10.3) to achieve the encapsulation of liquid precursors of biopolymers in the hollows of the mold; then, after solidification of the polymer, the mold can be gently peeled off from the substrate and thereby reduce demolding forces since the slight bending of the mold

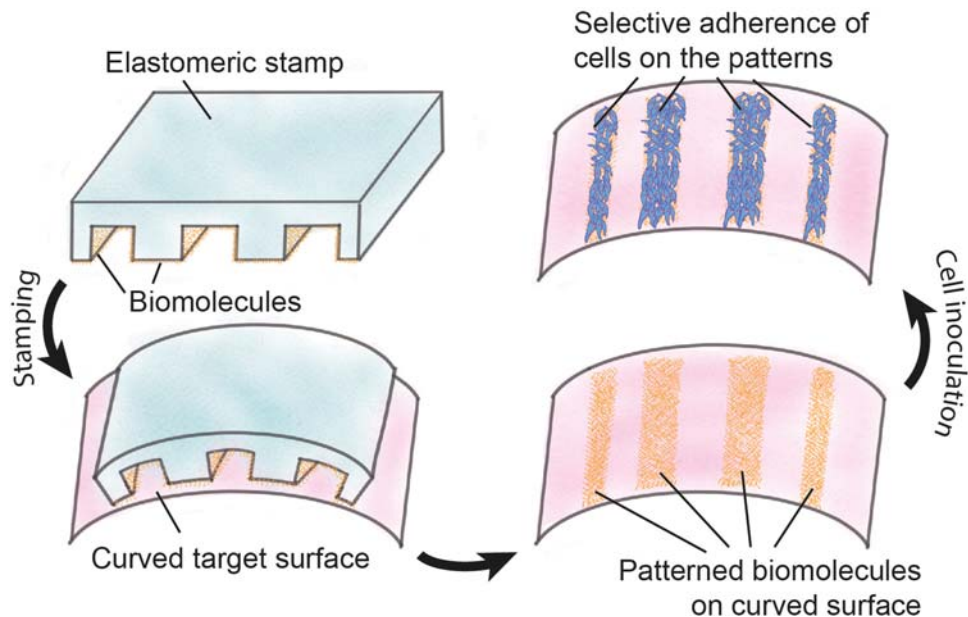
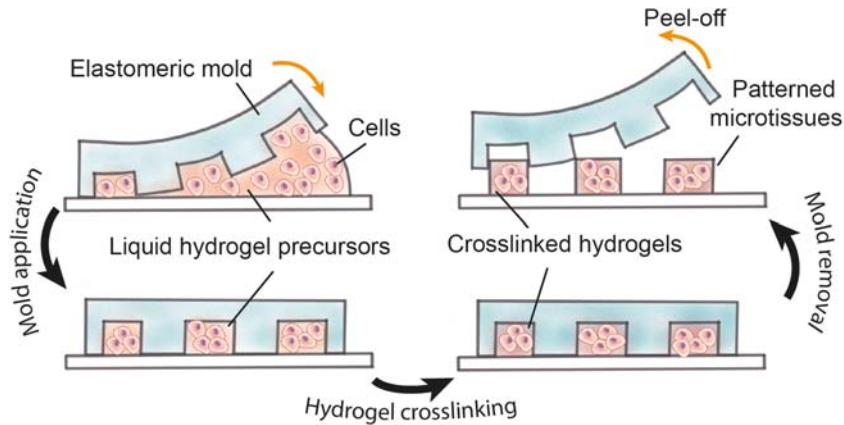


FIGURE 10.3

Schematic illustration of microcontact printing (μ CP) by the example of patterning cells on a curved surface.

**FIGURE 10.4**

Schematic illustration of a tissue micromolding technology to fabricate patterned microtissues.

gradually releases the surface tension that tightly holds together the mold and the substrate surface. As long as the mold is not reactive toward biomaterials and does not have any deleterious effect on the cells, micromolded tissue constructs can be developed using any fabrication scheme. For example, a myoblast-laden hydrogel sheet featuring ridged microstructures can be micromolded and assembled into skeletal muscle-like tissues in which the myotubes align with the direction of the stripe patterns. Such techniques can be used to fabricate muscle tissue-based robots⁹ (see 10.6 State-of-the-art experiment) and cultured meat.¹⁰

10.3.3 Microfluidic fabrication of microtissues

Microfluidics is the science and technology of systems that process or manipulate small (10^{-9} to 10^{-18} L) amounts of fluids mainly by confining the fluids using microscale structures, such as microchannels, chambers, etc. Since the fabrication of the microscale structures heavily relies on photolithography, soft lithography, etc., microfluidics seems to be merely a “spin-off” from these preexisting microfabrication techniques. In fact, microfluidics is not just about the fabrication of microchannels, chambers, etc.; microfluidics is also about the theory and approach to generating microscale patterns of static or dynamic flows in these microstructures by harnessing the unique behaviors of fluids at the microscale.

At the microscale, the forces that are related to the length or surface characteristics of the physical system (such as capillary or electrostatic forces, respectively) dominate over the forces that are related to the volume characteristics of the physical system (such as gravity or inertial forces). Imagine a micro-Galileo would try to drop his microball from a micro-Tower of Pisa. The ball would rather stick in the air than to quickly drop; the dragging force exerted by the air to stop the ball is related to the surface characteristic of the ball and therefore dominates over the gravity at the microscale. Specifically, for a biphasic fluidic system at microscale, adjacent flow streams of a pair of miscible fluids (such as aqueous solutions) tend to be neatly layered (i.e., laminar) rather than to be chaotic (e.g., to form vortexes) since the inertial forces of the fluidics are weak; for a pair of immiscible fluids (such as oil and water),

microdroplets as result of their emulsification can be generated with high uniformity in size that is close to monodisperse. To better understand the theoretical details of these microscale phenomena, several textbooks are recommended for further reading.

For tissue engineers, by incorporating cells and hydrogel precursors into microfluidic systems, microtissues can be fabricated with unprecedented control on their microscale shape and material composition to better mimic in vivo tissue morphologies and functions. Since the microtissues are featured with microscale critical dimensions (i.e., the minimum distance of the innermost cells to the tissue outer boundary) that can ensure sufficient oxygen and nutrient diffusion, the microtissues are easy to culture without the need for dedicated bioreactors.¹¹ Such microtissues can also be used as building blocks to be assembled into larger tissue constructs, which will be described in detail in [Section 10.2.4](#). Depending on their characteristic shapes, the microtissues can be categorized into mainly three types: point-, line-, and planar-shaped microtissues.

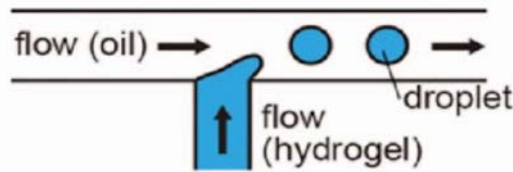
Microfluidic fabrication of point-shaped microtissues

Point-shaped microtissues take either a sphere or polyhedron shape that does not have an apparent long axis. Depending on their composition, the point-shaped microtissues can be further divided into cell spheroids and cell-laden hydrogel beads/particles: on the one hand, the cell spheroids consist of only cells and the matrix secreted by the cells; on the other hand, the cell-laden beads have a composition with a higher amount of hydrogel mimicking the in vivo ECM that can accommodate the cells and be remodeled by the cells during culture.

For the formation of cell spheroids, microcavities or hanging drops (i.e., drops of liquid suspended from a cover glass or the lid of a culture dish) can be used to confine the cells in a small volume to increase the chance of cell–cell contact. In addition, the hanging drops for the formation of spheroids can be connected with microchannels, which offers the opportunity to selectively “address” each spheroid in an array of microcavities and apply various types and concentrations of chemicals, increasing the flexibility of the high-throughput spheroid assay.¹²

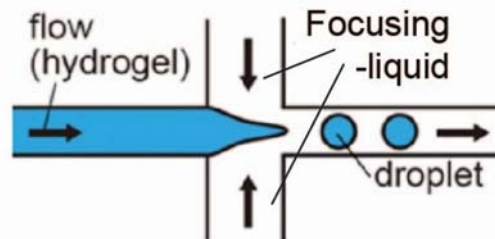
For the formation of cell-laden beads, emulsions of cell-laden hydrogel precursor in oil are first formed by flowing the cell-laden hydrogel precursor and oil into a microfluidic channel simultaneously. Then, the hydrogel precursor is allowed to cross-link, and the beads are transferred to culture medium for further cultivation. There are two typical microfluidic channel designs for the formation of microemulsions: T-junction channels and flow-focusing channels ([Fig. 10.5](#)). The T-junction channel has its name from its channel geometry resembling the capital letter T. In the T-junction channel, the oil is flowed from left side, while the cell-laden hydrogel precursor is introduced from the bottom side. When the liquids are tuned with optimized surface tension and injected at proper flow rates, an emulsion of cell-laden hydrogel will be formed at the crossing point and transported to the other side of the horizontal cross. In the flow-focusing channels, the channel geometry resembles a vertical cross. To form an emulsion, the cell-laden hydrogel precursor is introduced from the bottom of the vertical cross, and a “focusing-liquid” (e.g., oil) is introduced from both sides of the cross, which focuses and squeezes the cell-laden precursor into droplets and transports the droplets upward. It is also worthy of mentioning that the flow-focusing channel can take a 3D form, in which the focusing-liquid can be introduced from not only two opposite but all (circumferential) directions perpendicular to the axis of the flow of the

T-junction channel



Flow-focusing channel

Quasi-planar type



3-D (axial-symmetrical) type

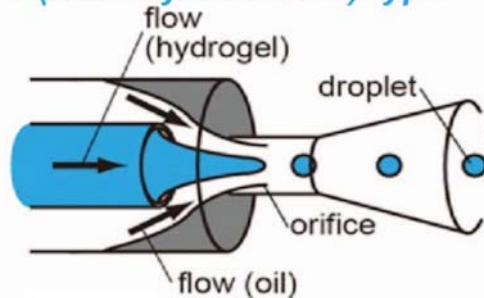


FIGURE 10.5

Two typical designs for the formation of microemulsions: the T-junction channels and flow-focusing channels. From Morimoto Y, Hsiao AY, Takeuchi S. Point-, line-, and plane-shaped cellular constructs for 3D tissue assembly. *Adv Drug Deliv Rev.* 2015;(95):29–39.

cell-laden hydrogel, resulting in more uniform focusing of the flow. The 3D flow-focusing channels can be fabricated by the assembly of multiple tapered glass capillaries or even high-resolution 3D printers. Based on the T-junction or flow-focusing channel design, also point-shaped microtissues with more sophisticated structures can be fabricated, such as the fabrication of core-shell structures by connecting the outlet of a flow-focusing channel to the inlet of another,¹⁴ and the generation of compartmentalized cell-laden beads by forming the emulsion of a multistreamed laminar flow.¹⁵

Microfluidic fabrication of line-shaped microtissues

Many of the in vivo tissues exhibit bundle-shaped morphologies in which cells take stretched shapes and are arranged in a highly aligned fashion, such as skeletal muscle and peripheral nerve. Line-shaped microtissues (a.k.a. “cell-laden (micro)fibers”) are long and thin tissues consisting of cells and hydrogels, which can provide geometrical cues to guide the elongation and function of cells. The microfluidic formation of line-shaped tissue requires the formation of fiber-shaped liquid threads and the solidification of the same.

First, for the formation of line-shaped liquid threads, it is typical to use the flow-focusing channels to produce point-shaped microtissues as described in the previous section while replacing the focusing fluid from an oil to an aqueous solution (Fig. 10.6). The cells can be either seeded on the surface of the microfibers, or preloaded within the hydrogel microfibers.

Since the aqueous solution is miscible with the cell-laden hydrogel precursor, a laminar flow pattern will be formed in which the two liquids separate into parallel streams. With an increasing ratio of the flow rates of the focusing fluid against the cell-laden hydrogel precursor, the liquid thread of the cell-laden hydrogel can be narrowed and eventually be fully surrounded by the focusing fluid, thus forming a line-shaped thread. The mixing of the cells or hydrogel precursor into the focusing fluid is dominated by diffusion and is slow for most application scenarios, for example, theoretically, the time for a neutrally buoyant 10 μm cell to diffuse itself across a quiescent 100 μm channel estimates to take approximately 30 years.

Second, it is important to solidify the line-shaped liquid threads before the flow stops. Solidification of the threads can be achieved by photo-cross-linking of the hydrogel precursors, as triggered by the light dosage, or the diffusional influx of ions. For light-initiated cross-linking, materials mentioned in a previous Section 10.3.1 are used with adjusted rheological properties (such as viscosity) in combination with flow rate optimization to achieve a stable formation of line-shaped threads with minimal damage

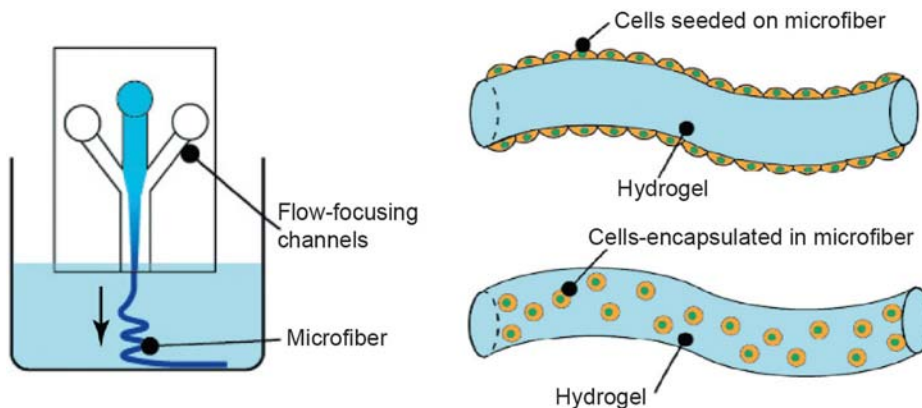


FIGURE 10.6

The use of flow-focusing channels to produce line-shaped microtissues by using aqueous fluids as the focusing fluid. From Onoe H, Takeuchi S. Cell-laden microfibers for bottom-up tissue engineering. *Drug Discov Today*. 2015;20(2):236–246.

to the cells; either too fast flow or too high viscosity can result in cell-damaging shear forces generated by the flow. For the diffusional influx of ions, the cross-linking is achieved by introducing the ions in the focusing liquid as cross-linker; once the two fluids contact, cross-linking will be triggered by the diffusion of ions into the hydrogel precursor, such as the diffusion of calcium ions into sodium alginate (sol) to form calcium alginate (gel). In addition, the cells can be either seeded on the surface of the microfibers, or as described above, suspended within the hydrogel microfibers (Fig. 10.6).

Due to the laminar flow regime of microfluidics, the composition and cross-section profile of the line-shaped tissues can be controlled with various channel designs, for example, multisectioned microfibers can be fabricated by connecting a multistreamed laminar flow as an input to the fiber-forming channels,¹⁷ core-shell microfibers can be fabricated by serially configuring the flow-focusing channels for the core and for the shell,¹⁸ and the composition along the fiber can be dynamically tuned by integrating a valve-controlled multiple stream-switching channel with the flow-focusing channel.^{19,20}

Microfluidic fabrication of plane-shaped microtissues

The most representative plane-shaped tissues are epithelial tissues, which are ubiquitous in the human body. Epithelia including endothelium line or cover the boundary between different tissues and fluid- or air-filled body lumens (such as in case of the lung, kidney, intestine, or blood vessels) or between the body and the outer environment (such as in case of the skin). In fact, the most popular cell culture method, i.e., the culturing of a monolayer of adherent cells in a culture dish can be considered as a method to form plane-shaped microtissue since the resulting cell monolayer has a planar shape and is microscale thin. However, the monolayer cannot be easily detached from the culture dish without disrupting the thin cellular sheet. Several methods solved the technical hurdle and can be considered the first generation of plane-shaped microtissues, such as the cross-linking of cell-laden hydrogel precursors in a Petri dish for the subsequent rolling-style assembly of vascular vessels²¹ or the detachment of the monolayer using temperature-responsive polymers grafted to the dish bottom (a.k.a. “cell sheets”), which have found applications in wound-healing or heart diseases.²²

With microfabrication and microfluidic techniques, the in-plane shape and composition of the plane-shaped microtissues can be controlled with microscale precision, such as by methods based on micromolding, photolithography, valving systems, and digital microfluidics. For the micromolding-based methods, a cell-laden hydrogel precursor is cast into a flat cavity with microfabricated spacing structures.^{23,24} By triggering the cross-linking of the hydrogel precursor (using a mist of calcium chloride for alginate or temperature for collagen), cell-laden hydrogel sheets can be fabricated with hollowed patterns as defined by the spacing structures. In addition, the handleability of the thin sheets can be enhanced by embedding a filter paper frame at the periphery of the sheets.²⁵ For the photolithography-based methods, first a cell-laden hydrogel precursor is flowed into a thin chamber, and then selectively cross-linked with patterned light; by washing out the uncross-linked hydrogel and refilling with another type of cell-laden hydrogel precursor, a sophisticated in-plane configuration of cells and hydrogels can be achieved.²⁶ For the valving system-based methods, the plane-shaped microtissues are fabricated by infusing multiple threads of cell-laden hydrogel precursors into a wide channel; there, they merge in wide streams and solidify into plane-shaped tissues. The planar composition of the plane-shaped tissues can be precisely controlled by programming the timing of each flow thread; such techniques have been used in conjunction with handheld bioprinters for in situ

application of plane-shaped tissues during surgery.²⁷ For the digital microfluidic-based methods, electrical potentials are utilized to dynamically change the wettability of a surface, which can be used to move small volumes of liquids (e.g., cell-laden hydrogel precursors) to a desired location within a plane; after placing each droplet in desired locations, light can be used to cross-link the hydrogel to achieve the fabrication of complex-patterned plane-shaped microtissues.²⁸

10.3.4 Microtissue assembly and application perspectives

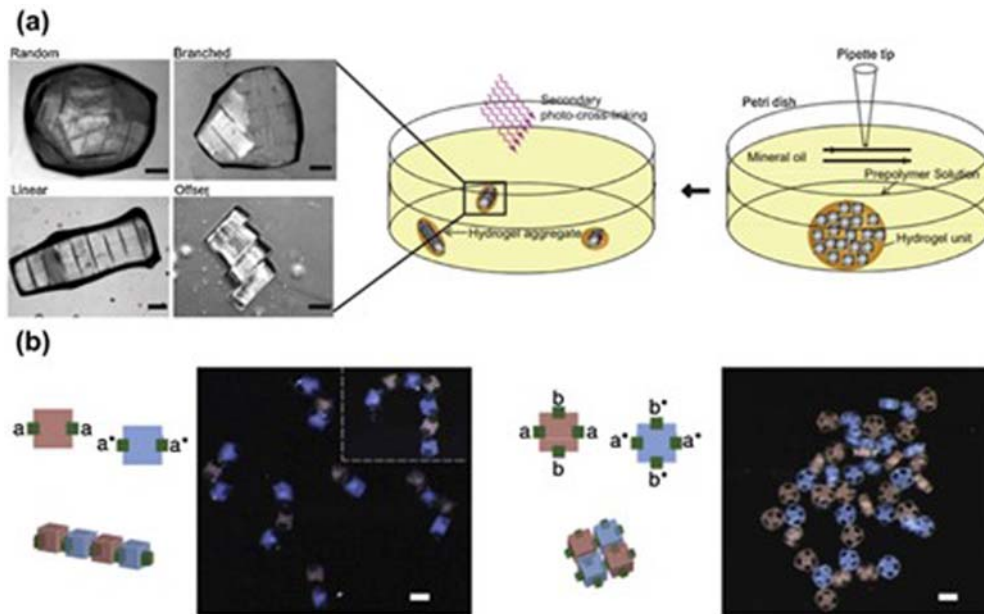
Histological studies have revealed that in vivo organs are structured in a hierarchical fashion; with the individual body as the top hierarchy, it can be divided into many lower hierarchical levels, such as organs, tissues, and cells, all the way down to the molecular level. With the capability of fabricating microtissues using microfabrication techniques, the microtissue-level serves as an emerging hierarchy level between the tissue level and cell level.

To engineer such a hierarchical system, in theory, there are two opposite strategies: *top-down* and *bottom-up*. On one hand, the *top-down* strategy first builds the blueprint/backbone of the top-level hierarchy and then deploys the components of the sublevel hierarchies. On the other hand, the *bottom-up* strategy approaches the task by first building a low-level hierarchy (a.k.a. “building blocks”) and performs level-by-level assembly to achieve the high-level hierarchy end product.

In practice, most fabrication methods are neither purely top-down nor purely bottom-up; the two strategies are often mixed together to yield the best empirical results that suit specific applications. For example, despite that the scaffold-based TE methods are commonly categorized as top-down as they build the scaffolds with defined shapes and then introduce cells into the scaffolds, the maturation of the tissue actually involves bottom-up characteristics since the cells will migrate, proliferate, differentiate, and form into microstructured aggregates to fill the gap between the cell-level hierarchy and the organ-level hierarchy from bottom-up. Having said so, it is still reasonable to tag the scaffold-based TE as top-down since the design of the scaffolds mostly dominates the macroscale outer shape of the end product, which is often important in clinical applications. Similarly, the microfabrication-based TE approaches can be tagged as bottom-up since mostly the fabrication starts with the patterning of a small number of cells and rely on the self-assembly of the cells to form into functional microtissues. In this section, we discuss several strategies for the assembly of the microtissues, which can be either bottom-up, such as the assembly of microtissues that utilize the local interactions between the microtissues, or top-down, such as textile techniques and templated assembly techniques, which arrange the microtissues according to a desired spatial distribution.

Self-assembly of microtissues

In order to induce their self-assembly, microstructures are often designed to present certain functional groups or molecules that can induce physical interactions (e.g., hydrophobic interaction, surface tension, capillary force), which drive their movements into intended rearrangements. For example, microgels (i.e., micro-sized hydrogel materials) suspended in hydrophobic solution could be assembled into larger constructs due to the high surface tension of the aqueous-based microgel suspension (Fig. 10.7a).²⁹

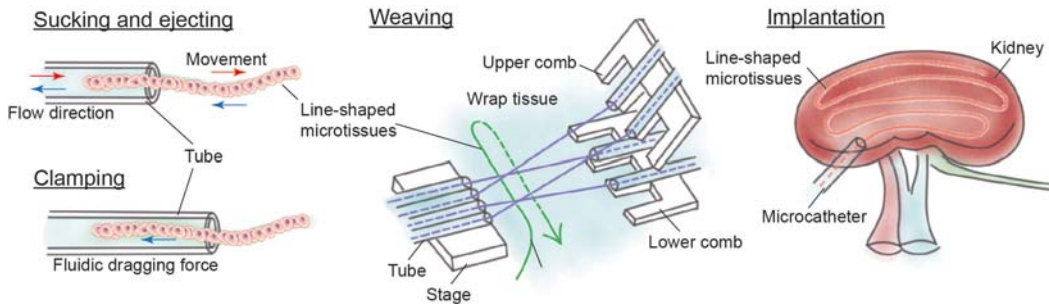
**FIGURE 10.7**

Self-assembly of tissue microstructures to form larger tissue constructs. Scale bar: 200 μm . From Qi H, et al. DNA-directed self-assembly of shape-controlled hydrogels *Nat Commun.* 2013;4:2275.

For its high specificity and strong attractive interaction, DNA base pairing could be demonstrated as a highly useful tool for inducing self-assembly. Khademhosseini and coworkers conjugated complementary DNA strands on two different sets of cell-laden microgels. These DNA strands were used as a “glue” to induce strong attraction between those two microgels to engineer larger aggregate tissue structures (Fig. 10.7b).³⁰

Textile techniques for the assembly of line-shaped microtissues

Textile technology deals with the fabrication, manipulation, and assembly of fiber-shaped (i.e., line-shaped) materials. Textile techniques can not only be used to weave cloths from cotton fibers but also to hold wounded tissues together with surgical sutures. By the adaption of current textile techniques to the line-shaped microtissues fabricated using microfluidic devices, the assembly of line-shaped microtissues can be achieved both in vitro and in vivo, producing functional 3D tissue models or therapeutic implants, respectively. Many traditional textile techniques have been adapted for the assembly of line-shaped microtissues and proven useful for this purpose, such as weaving, knitting, braiding, winding, or reeling.³¹ Since the line-shaped microtissues are made of hydrogels and cells, it is preferable to handle the microtissues in a liquid environment with optimized osmolarity, temperature, and pH. Instead of using mechanical tweezers or clamps to handle the line-shaped microtissues, fluidic methods, such as sucking and ejection of the line-shaped microtissues using plastic tubes, have been proposed to gently handle the living tissue (Fig. 10.8). The microfluidic manipulation utilizes

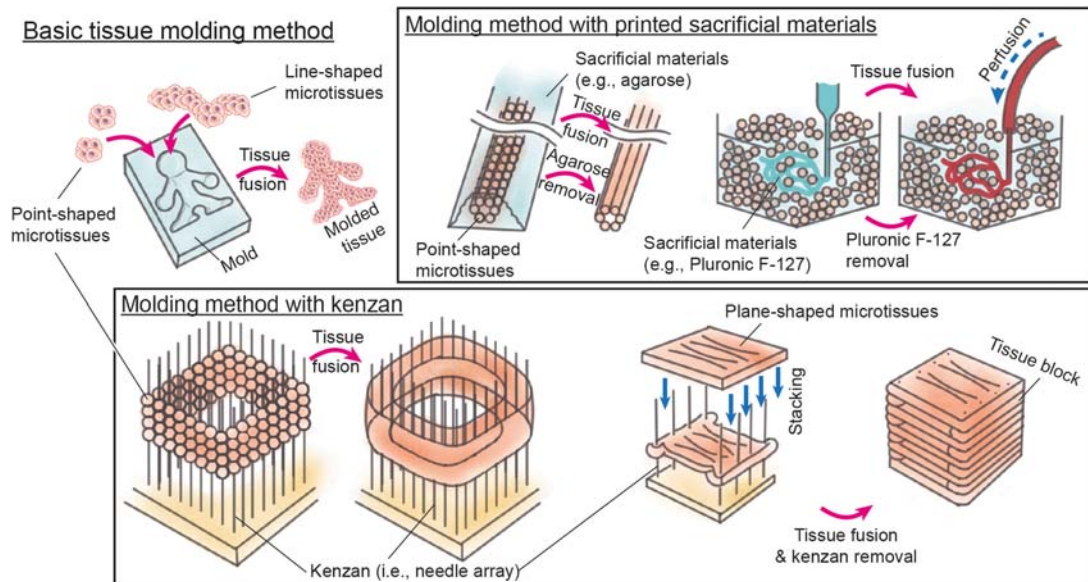
**FIGURE 10.8**

Textile technology in combination with the microfluidic manipulation of line-shaped microtissues (a.k.a. “cell-laden (micro)fibers”).

plastic tubes in combination with syringes to suck and eject the line-shaped microtissues with minimal damage to the cells. Clamping of the sucked line-shaped microtissue can be achieved due to the fluidic dragging force exerted by the fluid to the line-shaped microtissues to prevent the sucked line-shaped microtissues from escaping. By combining the microfluidic manipulation with the textile technique weaving, woven fabrics can be made with line-shaped microtissues and the whole weaving process can be performed in an aqueous environment. The microfluidic manipulation can also be implemented with a microcatheter to dispense the line-shaped microtissues into the *in vivo* tissue, e.g., into the sub-renal capsular space of a mouse kidney as illustrated. As a result, the islet cell-laden microfibers assembled *in vivo* can normalize the blood glucose concentration of the diabetic mice.¹⁸

Templated molding of microtissues

Microtissues can adhere to each other and undergo a process commonly referred to as “tissue fusion” in which the borders of the microtissues gradually merge to form a larger piece of tissue.³² Utilizing this phenomenon, as shown in Fig. 10.9, both point-shaped microtissues and line-shaped microtissues have been molded into 3D millimeter-scale constructs with their shape defined by a mold. Also, notice that the tissue molding methods discussed here refer to the assembly of microtissues into larger constructs, which differs from the creation of microtissues using tissue micromolding techniques as described in Section 10.2.2. To better define the shape and structure of the target construct, more complex templated molding methods are developed, such as the sacrificial printing methods and the KENZAN method. The sacrificial printing methods deposit a sacrificial material, such as carbohydrate glass, agarose, or pluronic F-127, before the microtissues are being deposited. By repeating this process in a layer-by-layer fashion, a structure supported by the sacrificial material can be constructed with a defined 3D shape. After the fusion of the microtissues, the sacrificial material will be removed by changing temperature to melt the sacrificial materials from solids/gels into liquids at cell/biomolecule-compatible temperatures. The KENZAN method takes inspiration from a technique used in the Japanese art of flower arrangement in which flower stems are plugged onto an array of vertical needles (a.k.a. “KENZAN”). During the process of the KENZAN method, point-shaped microtissues are picked and plugged onto the KENZAN using a pick-and-place robot, followed by fusion of the microtissues. Since the KENZAN helps holding the microtissue in defined positions, a 3D tissue construct with the desired shape and composition can be fabricated. The fabrication of implantable grafts by the KENZAN method, such as diaphragms³³ and nerve conduits,³⁴ has been demonstrated.

**FIGURE 10.9**

Templatd molding of microtissues using tissue fusion and line-shaped microtissues.

10.4 Future perspective

The ability to control the fabrication of a system and manipulate it on a micrometer scale is a highly innovative technology that is greatly coveted in all areas of research and industry. It offers several advantages as compared with bulk, macroscale systems: efficiency, reproducibility, high-resolution control, cost-effectiveness, miniaturization, and high throughput. The microfabrication technology was first driven by the remarkable technological advancements in semiconductor industry and its applications in microelectronics, fundamentally revolutionizing our way of life.

Many microfabrication processes involved in microelectronics, such as photolithography, have been the basis for the burgeoning affluence of current microfabrication technology, because these same processes are utilized to create tools needed to fabricate other devices. For example, the majority of MEMS devices are made by processing silicon wafers. In addition, PDMS elastomer-based microfluidic devices are made by casting and curing PDMS on silicon wafer-based masters typically prepared by photolithography. More recently, extensive research efforts are invested to combine several microfabrication technologies to develop more complex systems, such as for lab on chips or organ on chips by combining MEMS with microfluidics.

The ultimate goal of TE is to artificially engineer tissues or entire organs by encompassing the principles of biology, chemistry/material science, and engineering. Like any other research area involving material fabrication, TE has benefited tremendously from microfabrication technology in recent years. It is now well established that cells and tissues are regulated on the micro- and nanometer scales. Therefore, controlling the spacing, orientation, and overall architecture of a tissue-engineered construct on the micrometer scale would allow for the enhanced regulation of various cellular phenotypes. For this reason,

microfabrication technologies, such as photolithography, microfluidics, which have been originally developed for microelectronics and analytics, respectively, are now extensively investigated for a wide range of TE applications.

As the field of TE itself is considered by many to be still in its infancy, the utilization of microfabrication technology so far has been limited, and therefore not yet reached its true potential. As TE continues to expand and likely moves toward tissue-, organ-, and patient-specific strategies, this trend of utilizing microfabrication technology is expected to continue and eventually become a fixture in the future. This will be further fueled by the technological advancement that will render many microfabrication processes less expensive and more accessible, and therefore become more popular; do not look any further than the recent meteoric rise and popularity of 3D bioprinters.

Summary

- Microfabrication was conceptualized and came into fruition in the realm of electronics such as the fabrication of integrated circuits in semiconductor industry.
- Microfabrication technology is increasingly utilized in various TE applications, as it offers several advantages over conventional bulk methods: efficiency, reproducibility, high-resolution control, cost-effectiveness, miniaturization, and high throughput.
- In TE, photolithography is used to fabricate tissue-engineered hybrid constructs from cells and photo-cross-linkable ECM-mimicking hydrogels with micrometer-scale resolution and dimensions by controlling the patternable area with a photomask, which has laid the foundation of rapid prototyping (RP) techniques such as stereolithographic bioprinting.
- Soft lithography has extended the capability of photolithography especially in the patterning of biomaterials. Also, the introduction of elastomers such as PDMS into biological labs has made it easy for bioengineers to access and benefit from microfabrication.
- The size and shape of tissue-engineered constructs can be easily tuned by using a patterned substrate as a micromold.
- A desired pattern of biological molecules (e.g., proteins, peptides, and DNA) can be transferred onto a substrate using μ CP.
- Microfluidic technology allows the control of the flow of small amounts of fluids through micrometer-scale channels, which have led to the high-throughput fabrication of point-, line-, and plane-shaped microtissues.
- Microfabricated structures can be used as building blocks that are assembled into larger, hierarchical structures.

Classical experiment

This experiment aimed to fabricate 3D muscle tissues with highly aligned myotubes utilizing microfabricated grooves.⁶ First, a piece of PDMS slab with micropatterned wavy surface was prepared by stretching the PDMS slab mechanically, oxidizing it in an oxygen plasma machine, and subsequently allowing it to relax to squeeze the oxidized

surface to form wavy patterns. Then, the PDMS slab was placed in a 35 mm plate, coated with laminin, and pinned with two braided sutures (length 6 mm, precoated with laminin to promote cell adhesion). Next, primary skeletal muscle cells were plated onto the wavy surface of the PDMS. The microscale wavy pattern on the surface of the PDMS

Continued

Classical experiment—continued

slab serves as a geometrical cue to guide the alignment of the differentiated skeletal muscle cells. When ca. 80%–90% of the myoblasts differentiated and fused into myotubes, fibrin gel was formed on top of the cell layer. During subsequent culture, the cells gradually digested the fibrin gel and the laminin substrate, leading to the detachment of the fibrin gel substrate with the cells incorporated within the gel. Further culture of this detached cell-containing fibrin gel, anchored by the two braided sutures, led to the

rollup of the gel into rod-shaped tissue with the two sutures acting as anchoring points at the two ends of the tissue rod. The alignment of the myotubes, engineered by geometrical cues of the wavy pattern of the PDMS slab, was successfully preserved in the tissue rod after the rollup process, leading to the fabrication of a 3D muscle tissue with highly aligned myotube structures, resembling the native morphology of skeletal muscle tissues (Fig. 10.10).

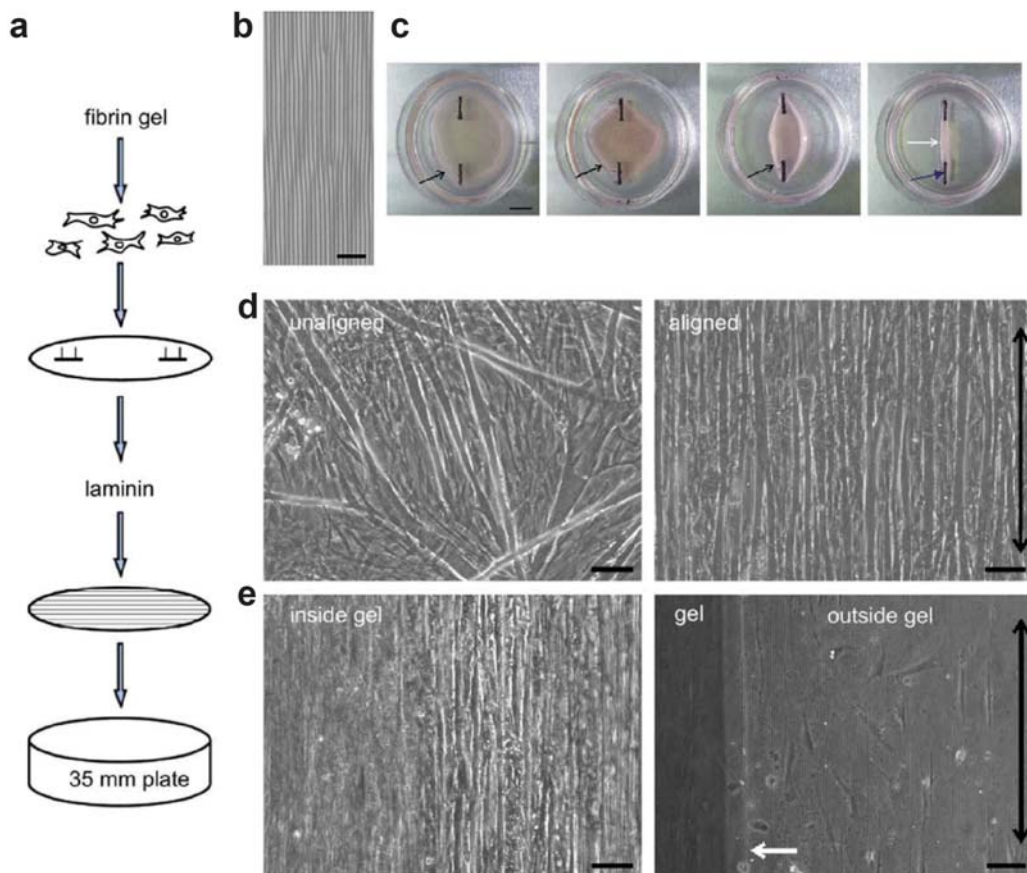


FIGURE 10.10

Formation of 3D tissue with highly aligned myotubes using microfabricated wavy surface patterns: (a) Diagram of tissue formation method. (b) Microscopic images of the wavy surface pattern on the PDMS slab. (c) Rollup process of the 3D tissue. (d) Images of cells cultured on flat versus wavy PDMS. (e) Images of cells on the wavy features inside and outside the gel. Scale bars: (b) 50 μm; (c) 6 mm; (d, e) 100 μm. From Lam M, et al. *Microfeature guided skeletal muscle tissue engineering for highly organized 3-dimensional free-standing constructs*. *Biomaterials*. 2009;30(6):1150–55.

State-of-the-art experiment

This experiment aimed at fabricating a biohybrid robot powered by engineered 3D skeletal muscle tissues with highly aligned myotubes [Fig. 10.11].⁹ The robot consists of two pieces of skeletal muscle tissues, artificial skeletons consisting of anchors, a joint, flexible ribbons, and pairs of electrodes. The robot can generate bidirectional joint motion by selectively applying electrical stimulation to the muscle tissues, which will contract and pull the flexible ribbon toward the desired direction.

The fabrication of the robot starts from preparing the artificial skeleton. Components of the artificial skeletons,

such as anchors with arrays of micropillars, flexible ribbons, and pairs of electrodes, were prepared using stereolithography and photolithography, respectively. The components were assembled using UV adhesives. Then, tissue micromolding method was utilized to mold thin myoblast-laden hydrogel sheets with holes and striped structure. Next, the hydrogel sheets were stacked with the holes threaded through the micropillars of the anchors and cultured subsequently for the differentiation of the myoblasts.

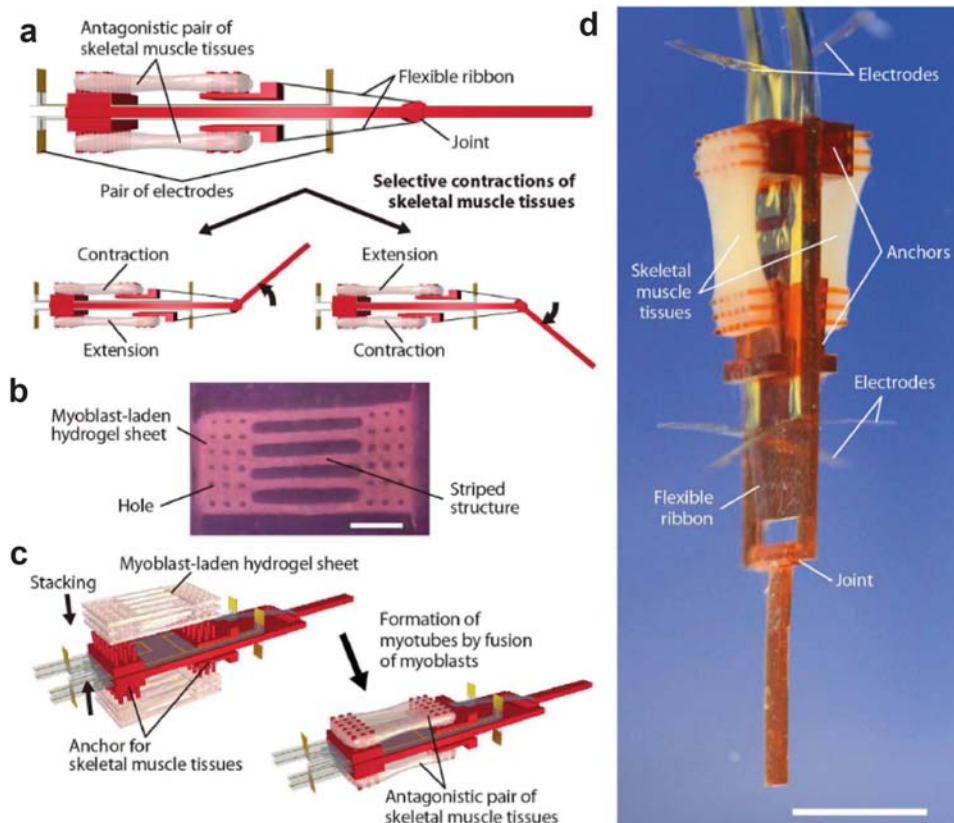


FIGURE 10.11

Biohybrid robot powered by skeletal muscle tissues: (a) Scheme of the robot. (b) The myoblast-laden hydrogel sheet fabricated using tissue micromolding. (c) Formation of the muscle tissues on the skeleton of the robot. (d) Photo image of the robot. Scale bars: (b) 2 mm; (d) 5 mm. From Morimoto, et al. *Biohybrid robot powered by an antagonistic pair of skeletal muscle tissues*. *Sci Robot*. 2018;3(18):eaat4440.

10.5 Recommended literature

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10.6 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
1. What is MEMS?
 2. Can you recall one to two applications enabled by MEMS technology in your daily life?
 3. What is the purpose of a cleanroom?
 4. What is the volume range that microfluidic system aims to control?
 5. What is μ TAS?
 6. What is PDMS?
 7. Can you recall the four steps of the general sequence for obtaining micropatterned PDMS?
 8. Explain the etymological meaning of “photolithography”.
 9. What is a photomask?
 10. How to use photomask to generate patterns on a thin film made of photoresist (PR)?
 11. What is the difference between positive and negative PRs?
 12. Explain the concept of soft lithography. How is soft lithography related to photolithography?
 13. What is the dominant flow regime in microfluidic systems?
 14. Explain microcontact printing technique and how the technique can be used to pattern cells.
 15. What is tissue micromolding?
 16. Explain the advantages of microtissues.
 17. How can microbeads/droplets be formed using T-junction channels?

18. What is the configuration of flow-focusing channels?
 19. Recall two to three typical types of physical interactions that are useful for self-assembly of microtissues.
 20. Explain the role of sacrificial material in the templated molding of microtissues.
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations.
1. Since most of the photoresist reacts to UV lights, what is the preferred setup for room lights for performing photolithography? Is there any possible strategy to perform photolithography without the worry of unwanted photo cross-linking under daylight?
 2. For traditional monolayer culture of anchorage-dependent cells using cell culture dishes/flasks, the liquid level is strictly controlled to be lower than ca. 1–2 mm to ensure sufficient oxygen delivery from ambient environment to the cells. Think about the case of culturing cells in enclosed microchannels, what are the possible strategies to deliver oxygen to the cells? (Hint: think about how human body tackles this issue; check the oxygen permeability of various materials such as silicon, glass, PDMS, etc.)
 3. Following the previous question. Think about strategies to deliver sufficient nutrients and oxygen to the cells in the large tissue constructs assembled using the point-, line-, plane-shaped microtissues.
 4. Learn the concept of Reynolds number in fluidics. Calculate the Reynolds number of the microfluidic channel.
 5. What is the advantage of the laminar flow regime to pattern biomaterials? Is it possible to generate chaotic flow in microfluidic systems and how?
 6. Think about a sequence of experiment in your own project, can it be fulfilled in a one-stop fashion using μ TAS? (Hint: check out the microfluidic cell sorting machines as an example)
 7. Is it possible to increase the throughput of biological assays using microfluidic systems and how?
 8. How can microfabricated electrodes be used for tissue engineering purposes?
 9. Conceive a strategy to build a textile machine to weave line-shaped microtissues.
 10. Can the microfluidic methods for the fabrication of point-shaped microtissues also be used to fabricate artificial cells? If so, how could these artificial cells be used for tissue engineering and regenerative medicine?
 11. Consider how to engineer perfusable lumen structures using microfabrication techniques, and how the angio/vascular-genetic ability of endothelial cells such as human umbilical vein endothelial cells (HUVECs) could be combined with microfabricated channels to create perfusable vasculature networks.

Challenge based learning

Microfabrication of an antithrombotic surface topography on stents

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Biomaterials are employed in the manufacturing of medical implants and as scaffolding materials in tissue engineering and regenerative medicine. Human cells and tissues frequently elicit an unwanted response, such as encapsulation, implant loosening, or stress shielding which can lead to implant failure. Micropatterning at the nanometer and micrometer range has been used in this field to influence cell–material response and it is the long-term vision to engineer custom-designed biomaterial topographies onto medical implants to manipulate cell response for an optimal function.

Motivation and stakeholders

Patients with occluded blood vessels are treated with implanted stents which restore the blood flow. A disadvantage of stents is that their materials induce blood clotting. As a consequence, patients with stents need to use blood-thinning medicine their whole life, which bears risks and is costly. Solutions to mitigate this problem should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as cardiovascular surgeons, biomaterial engineers, and entrepreneurs who have to implement new stent technology in cost-effective products.

Problem definition

Currently, photolithography is a 2D technique, whereas a stent is a 3D structure. Assuming that future biomaterial screening efforts will lead to the identification of topographies that induce reendothelialization, this still leaves the question on how to put them on a stent. Therefore, there is a clinical need to generate microfabrication strategies

that introduce nano- and microtopographies in anticlotting stents.

Challenge

To design a microfabrication technology that could introduce anticoagulant topographies in the stents currently used in the market.

Learning framework

Reading the Microfabrication Technologies and Cardiovascular Tissue Engineering chapter will help you to understand the following:

1. The clinical implication of atherosclerosis.
2. The mechanisms of plaque build-up in blood vessels
3. The current treatment strategies for occluded blood vessels
4. The ways biomaterials induce coagulation and the pharmacological interventions to resolve clots in blood vessels.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

5. Current strategies to manufacture topographies on 2D surfaces.
6. The state-of-the-art of to produce topographies on 3D surfaces.
7. The current strategies to induce reendothelialization in stents.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

10.7 Glossary

Artificial extracellular matrices are the engineered biomaterials that partially or fully mimic the composition, structure/morphology, and function of the in vivo extracellular matrices.

Biopolymers are long chain molecules produced by the cells of living organisms.

Cleanrooms are facilities designed to maintain extremely low levels of particulates, such as dust, airborne organisms, or vaporized particles, and used for specialized industrial production or scientific research.

Etching masks are films on top of substrates with predefined patterns, which are more resistant to specific etchants than the substrates, for the selective removal of substrate materials according to the patterns.

Lab-on-a-chip is a device that integrates one or several laboratory functions on a circuit called a “chip”) of a few square centimeters.

Micro Total Analysis Systems (μ TAS) describe microscale systems that include and automate all necessary steps for chemical analysis of samples, e.g., sampling, sample transport, filtration, dilution, chemical reactions, separation and detection.

Micro-Electro-Mechanical Systems (MEMS) are microscaled systems consisting of miniaturized mechanical and electromechanical elements made by microfabrication techniques.

Microactuators are microscopic devices capable of generating mechanical motion of solids or fluids.

Microcontact printing is a type of soft lithography procedure where a microstructured elastomeric stamp is inked with a material solution to be then deposited by direct contact with the surface of the substrate.

Microenvironments are the micrometer range environments of cells.

Microfabrication is a process that consists of designing, production, and characterization as well as application of patterns/structures, devices, and systems at the micrometer scale.

Microfluidics is the science and art on studying and engineering the behavior of small (often less than cubic millimeters) amounts of fluids.

Mix-pour-cure-peel sequence is a sequence of PDMS microstructure fabrication.

Photo(-reactive) resins are polymeric resins the solubility of which in the solution of a subsequent “development” step changes when exposed to light, often in the ultraviolet or region.

Photolithography is a process used to create patterns on a photosensitive polymer film by selective exposure to light through a mask and subsequent “development” using chemical agents.

Photomasks are plates used in photolithography with selective, patterned transparencies to allow light to pass selectively.

Polydimethylsiloxane (PDMS) is a widely used elastic polymer used for the fabrication of microfluidic devices or molds, stamps, or masks for soft lithography.

Soft lithography is a set of techniques used to fabricate micro- or nanoscale structures by molding or transfer of materials onto substrates using elastomeric, “soft” molds or stamps.

Tissue micromolding is a process where biopolymers are molded into tissue-like shapes.

© Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Scaffold design and fabrication

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11.1 Learning objectives

After reading this you will be able to:

- Learn the “4F” concept of scaffold design.
- Recognize the diversity of scaffold processing techniques, their advantages and disadvantages.
- Understand the dynamics of cell material interaction, integration of biomaterials in scaffold design and degradation of biomaterials.
- Gain an understanding of additive, formative, or subtractive manufacturing technologies.
- Be familiar with the use of scaffold design in personalized medicine.

Embrace complexity. Engineer versatility. Deliver simplicity.

Glenn Prestwich

Tissue Regeneration is not a 100-meter sprint but a Triathlon. In the past, a large group of scientists and engineers rationalized that scaffolds should be designed to have the exact mechanical and structural properties as the native tissue extracellular matrix that needs to be regenerated. However, looking at the developmental biology and the regeneration of tissues more closely, it is clear that not only the cells but also the extracellular matrix undergoes many iterations before the tissue is repaired and/or regenerated. Hence, the aim for scaffold design should be to guide the complex process and not base the design on the healthy tissue.

Dietmar W Hutmacher.

11.2 Introduction

Scaffold-guided tissue engineering (SGTE) uses a three-dimensional (3D) structural “substrate” to instruct cells and tissues to guide regeneration of tissues. The challenge is to translate the knowledge gained from *both developmental biology and natural tissue regeneration* toward an informed scaffold design which can guide cell and tissue organization in a predetermined tissue regeneration direction. In order

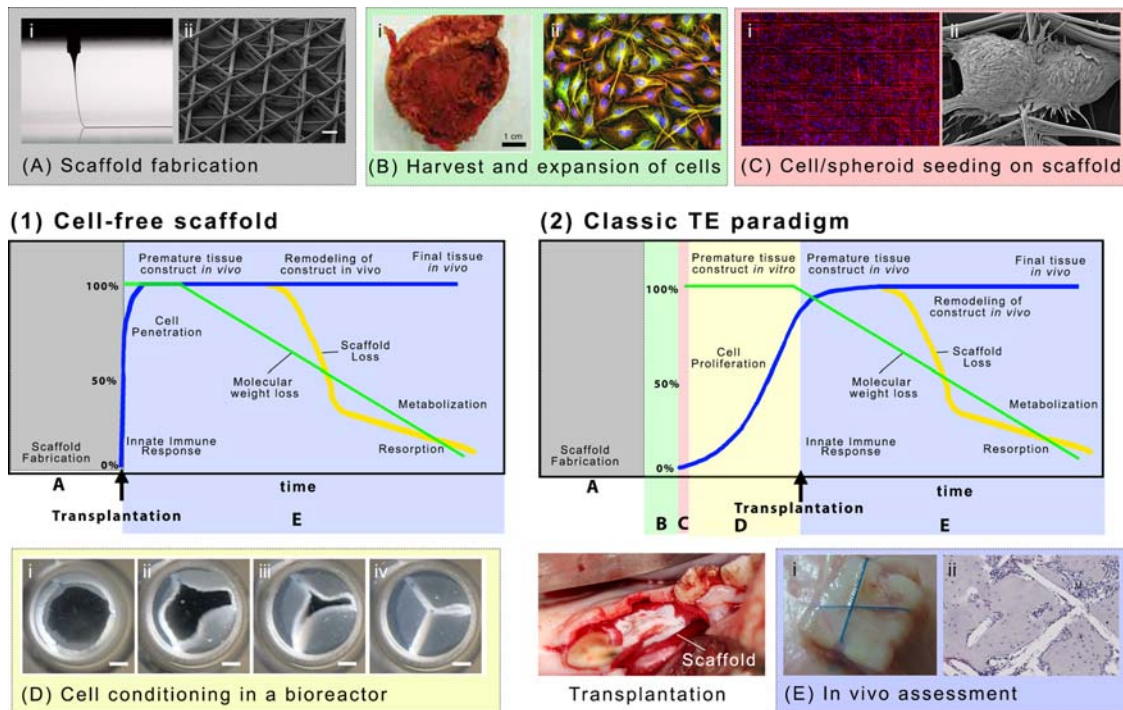
to reach this goal, a concerted, systematic effort is necessary to study the cellular, biochemical, and mechanoresponsive sequences for the entire cascade of tissue regeneration within scaffold designs.

The design palette for scaffolds is expansive, involving an infinite combination of surface chemistries, morphologies, resolutions, and mechanical properties, all changing with time. The quest is not to replicate/copy the physical structure of the tissue to be regenerated but to guide the regeneration process by designing an architecture that allows, with or without additional biological stimulation (in form of peptides, growth factors, etc.), cell migration, proliferation, and subsequent vascularized tissue formation. The scaffold should have a tissue-specific design which stimulates and directs the various stages of tissue regeneration from the initial host response toward the creation of functional tissue. Scaffolds promote new tissue formation by providing both a surface and void volume that promotes connective tissue, where a new one is needed. Essential variables in scaffold design and function include the **bulk material** or biomaterials from which it is made, the 3D architecture (morphology), the surface chemistry (where cell–material interactions occur) and the mechanical properties, including when the neo-tissue takes on the load.

Scaffolds have biochemical, chemical, and physical requirements, which depend on the specific tissue and strategy used. When implanted, they often must provide sufficient initial mechanical strength and stiffness to substitute for the mechanical function of the diseased or damaged tissue, which it aims at repairing or regenerating. Scaffolds may not necessarily be required to provide complete mechanical equivalence to healthy tissue, but the transmission of forces should be taken into consideration. For example, a scaffold for skin TE (see [Chapter 15 Skin engineering and keratinocyte stem cell therapy](#)) should allow for suture retention over 2–4 weeks and be able to withstand the **wound contraction** forces. In the case of scaffold-based bone TE, external and internal fixation systems might be applied to support the majority load bearing forces until the bone has matured.¹

Combining a scaffold with cells/tissue in vitro and/or in vivo is termed here as a **tissue engineering construct** (TEC). Cell and tissue remodeling is key for achieving stable biomechanical conditions and vascularization at the host site. Hence, the TEC should maintain sufficient structural integrity during the in vitro and/or in vivo growth and then remodeling process. The degree of remodeling depends on the tissue itself, and its host anatomy and physiology. When implanted, the scaffold morphology may need to allow a stable fibrin network, or control cell migration into and through it, mass transfer of nutrients and metabolites and provision of sufficient space for development and later remodeling of organized tissue. The degradation and resorption kinetics of the scaffold need to be designed based on the relationships of mechanical properties, molecular weight, **mass loss**, and tissue development (including vascularization) schematically shown in [Fig. 11.1](#). If the scaffold degrades too quickly, the strategy will fail. If it takes too long for the scaffold to degrade, fibrosis can occur which also will deleteriously affect the regenerated tissue.

[Fig. 11.1](#) schematically shows the interdependence of tissue penetration (blue line), molecular weight loss (green line), and mass loss (yellow line) of the scaffold with time. This degradation time frame impacts the TEC integration and remodeling toward complete resorption of the scaffold. When a cell-free scaffold is implanted in vivo, the interactions with blood—including hematoma formation—and the innate immune reactions occur rapidly. This sequence of events occurs in a similar way for a TEC which has been conditioned in a bioreactor. Vascularization results over the next several weeks

**FIGURE 11.1**

Two biodegradation timelines for a TEC. (1) Cell-free scaffold used directly as an implant or (2) the classic TE paradigm that uses a bioreactor to modulate a cell-seeded scaffold that is later implanted. Examples in a–e are for a spectrum of tissues, but all are made with the same scaffold fabrication technology. (1) and (2) Adapted from Henkel J, Huttmacher DW. *Design and fabrication of scaffold-based tissue engineering*. Bionanomat 2013;14(3–4):171–193. a(i) From Youssef A, Hrynevich A, Fladeland L, et al. *The impact of mels electrowritten scpfold deign on porosityrdetermined by X-Ray microtomography*. Tissue Eng C Methods 2019;25(6):367–379. b(i) Adapted from Martine LC, Holzapfel BM, McGovern JA, et al. *Engineering a humanized bone organ model in mice to study bone metastases*. Nat Protoc 2017;12(4):639–663. b(ii) From Gerardo-Nava J, Fuhrmann T, Klinkhammer K, et al. *Human neural cell interactions with orientated electrospun nanofibers in vitro*. Nanomedicine 2009;4(1):11–30. c(ii) From McMaster R, Hoefner C, Hrynevich A, et al. *Tailored melt electrowritten scaffolds for the generation of sheet-like tissue constructs from multicellular Spheroids*. Adv Healthcare Mater 2019;8(7):1801326. (d) From Saidy NT, Wolf F, Bas O, et al. *Biologically inspired scaffolds for heart Valve tissue engineering via melt electrowriting*. Small 2019;15(24):1900873. e(i) From Vaquette C, Fan W, Xiao Y, Hamlet S, Huttmacher DW, Ivanovski S. *A biphasic scaffold design combined with cell sheet technology for simultaneous regeneration of alveolar bone/periodontal ligament complex*. Biomaterials 2012;33(22):5560–5573. e(ii) From Thibaudau L, Taubenberger A, Holzapfel BM, et al. *A tissue engineered humanized xenograft model of human breast cancer metastasis to bone*. Disease Models Mech 2014;7(2):299–309.

and new tissue forms and is remodeled around the scaffold struts, often over many months. The graphical illustration of Fig. 11.1 shows that the scaffold material must be selected and/or designed with a degradation and resorption rate such that the strength of the scaffold is retained until the regenerated tissue inside the available pore spaces is fully remodeled by the host tissue and can assume a progressively increasing structural role. This mechanical stability of the scaffold throughout the implant

lifetime is essential; if the integrity is compromised, the biomechanical loading will jeopardize the implant. For long-degrading scaffolds, fibrous encapsulation may result in failure of the construct to truly regenerate the defect site. Hence, a key requirement of the scaffold design must be that the physical support by the scaffold itself is maintained until the engineered tissue within the interconnected pore morphology has been remodeled and built-up sufficient mechanical integrity to support the major loading profile in the former defect area. A common issue with TE is that scaffolds degraded too rapidly before the tissue had an opportunity to mature.

A holistic TE strategy must consider the practical considerations of manufacturing to allow translation from bench to bedside.^{2,3} From a clinical perspective, a scaffold must be manufactured under GMP (Good Manufacturing Practice, see [Chapter 20 Product and process design: toward industrial TE manufacturing](#)) conditions in a reproducible and quality-controlled fashion at a cost which would allow the marketed product to be affordable for the health care systems. To address this, advanced manufacturing processes is one major difference between the scaffolds of the 21st century and those of the 1990–2000s. This chapter provides an overview of the methods that are both historically and currently relevant for scaffolds studied in the field of tissue engineering (TE).

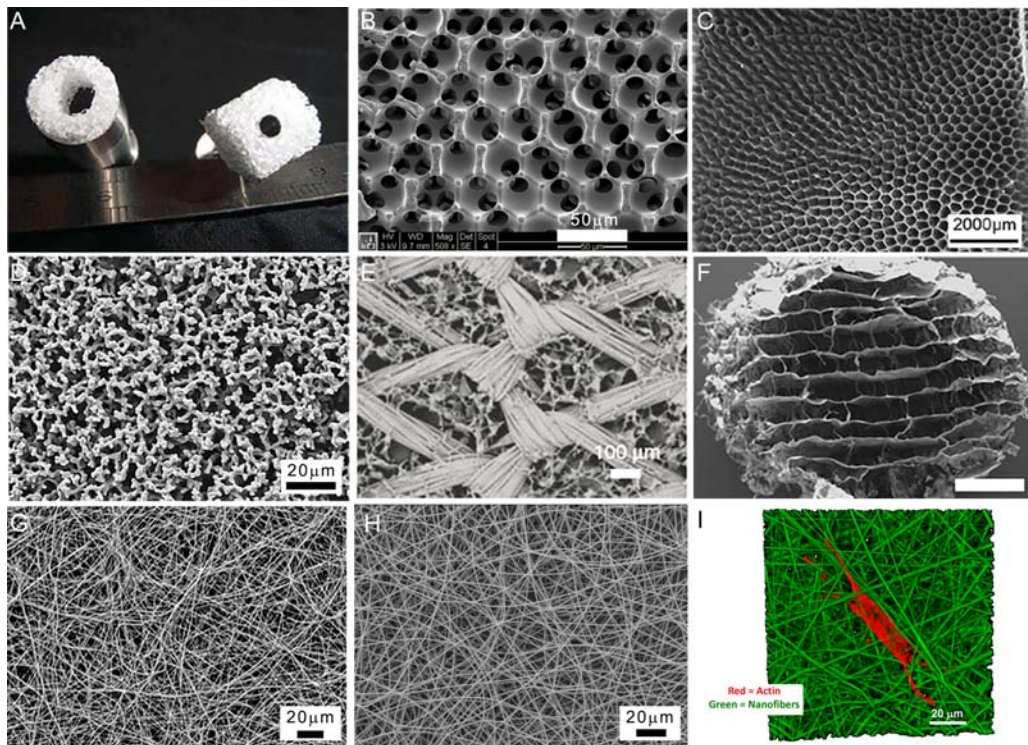
11.3 Scaffold design

[Fig. 11.1](#) shows that the scaffold design is complex and includes the material composition, morphology (including porosity), structural mechanics, surface properties, degradation properties, and by-products, together with the composition of any added biological components.⁴ The in vivo scaffold design can be remembered with the “4F concept”—that a scaffold should have a suitable *form*, temporarily support tissue *function*, guide tissue *formation*, and all delivered in a manner so that a surgeon can perform *fixation*. These aspects are different based on the anatomical and physiological features of the species and tissue to be regenerated. For example, the pore size and size of pore interconnections for the TE of mice bone should not be greater than 100 microns. In contrast, in large animals such as sheep and pig, these parameters must be >500 microns. Hence, TE concepts in humans are typically based on results derived from appropriate large animal models.

Since TECs will eventually be implanted, they should be nonantigenic, noncarcinogenic, nontoxic, non-teratogenic, and possess high cell/tissue biocompatibility so that they will not trigger pathological reactions after implantation. Scaffolds may be required to provide sufficient initial mechanical strength and stiffness to substitute for the mechanical function of the diseased or damaged tissue which the TEC aims at repairing or regenerating. Tissue remodeling is an essential period for achieving stable biomechanical conditions and vascularization within the host site. The porosity and internal space within a degradable scaffold will increase with time, allowing increased space for tissue to develop and/or remodel ([Fig. 11.1](#), see also [Chapter 7 Degradation of biomaterials](#)).

11.3.1 Morphology

Scaffolds have morphologies that relate to the fabrication process, and some classical examples are shown in [Fig. 11.2](#). In most biomechanically challenging applications, it is likely that the test for the tissue engineer will be to achieve sufficient stiffness and strength in a highly porous structure to provide adequate mechanical integrity. One of the most demanding applications will be the repair and

**FIGURE 11.2**

Selection of classical scaffold examples. (a) Photograph of salt porogen-leached PLGA, (b) SEM of a porogen-leached hydrogel (c) micromolded scaffolds used for spinal cord injury (d) randomly organized pore morphology of a scaffold made via polymerization-induced phase separation. (e) Braided yarns macropore architecture combined with a micropore filling collagen matrix. (f) Channeled pores made via ice templating. (g) A nonwoven fabricated via solution electrospinning, (h) melt electrospinning and (i) confocal microscope image of a cell growing on a solution electrospun membrane. (b) From Bryers JD, Giachelli CM, Ratner BD. *Engineering biomaterials to integrate and heal: the biocompatibility paradigm shifts*. *Biotechnol Bioeng* 2012;109(8):1898–1911. (c) From Lynam D, Bednark B, Peterson C, Welker D, Gao MY, Sakamoto JS. *Precision microchannel scaffolds for central and peripheral nervous system repair*. *J Mater Sci Mater Med* 2011;22(9):2119–2130. (d–g) From Li HY, Fuhrmann T, Zhou Y, Dalton PD. *Host reaction to poly(2-hydroxyethyl methacrylate) scaffolds in a small spinal cord injury model*. *J Mater Sci Mater Med* 2013;24(8):2001–2011. (e) From Chen GP, Sato T, Ohgushi H, Ushida T, Tateishi T, Tanaka J. *Culturing of skin fibroblasts in a thin PLGA-collagen hybrid mesh*. *Biomaterials* 2005;26(15):2559–2566. (f) From Bozkurt A, Lassner F, O'Dey D, et al. *The role of microstructured and interconnected pore channels in a collagen-based nerve guide on axonal regeneration in peripheral nerves*. *Biomaterials* 2012;33(5):1363–1375. (h) previously unpublished. (i) From Kumar G, Tison CK, Chatterjee K, et al. *The determination of stem cell fate by 3D scaffold structures through the control of cell shape*. *Biomaterials* 2011;32(35):9188–9196.

generation of musculoskeletal tissues, particularly bone, where scaffolds need to have a high elastic modulus in order to provide temporary mechanical support without showing symptoms of fatigue or failure, to be retained in the space they were designated for and to provide the tissue with adequate space for growth.¹ One of the fundamental challenges of scaffold design and materials selection is that

to achieve sufficient strength and stiffness, the scaffold material must have both a sufficiently high interatomic and intermolecular bonding and/or a physical and chemical structure which allows for hydrolytic attack and breakdown.

11.3.2 Porosity

A pore can be defined as a void space within a scaffold, whereas porosity can be considered as a collection of pores. Pore size and porosity are two of the most important scaffold parameters.⁵ Macropores (i.e., $>50\ \mu\text{m}$) are of a scale to influence tissue function. Micropores (i.e., $<50\ \mu\text{m}$) are of a scale to influence cell function (e.g., cell attachment) given that mammalian cells typically are $10\text{--}20\ \mu\text{m}$ in size. Nanoporosity refers to pore morphology or surface textures with features less than a micron (i.e., $1\text{--}1000\ \text{nm}$). There is often a compromise between porosity and scaffold mechanical properties. High porosity (e.g., 90%) may provide a greater pore volume for cell infiltration and extracellular matrix (ECM) formation, but conversely decreases mechanical properties in accordance with a **power-law relationship**.

11.3.3 Interconnectivity

Pore interconnectivity is essential, and surprisingly overlooked in classical scaffold design and characterization from the 1990s. A scaffold may be porous, but unless the pores are interconnecting (i.e., voids linking one pore to another), they serve no purpose and are superfluous. The pore interconnections should be suitably large to support cell migration and proliferation in the initial stages and consequent ECM infiltration of desired tissue. It is preferable that scaffolds have 100% interconnecting pore volume, thereby also maximizing the diffusion and exchange of nutrients (e.g., oxygen) throughout the entire scaffold pore volume. Fiber-based scaffolds have inherently interconnected pores; however, they are only truly cell invasive at a scaffold thickness $>1\ \text{mm}$ above a diameter of $10\text{--}20\ \mu\text{m}$ when they are produced as a random nonwoven.⁹

11.3.4 Pore characterization

As a measure of pore interconnectivity, the accessible pore volume, or permeability, of a scaffold can be quantified. Techniques such as mercury intrusion porosimetry, microcomputed tomography (μCT), or image analysis^{10–13} are used in this context. Mercury porosimetry is a popular technique which is based on the principle that the pressure required to force a nonwetting liquid such as mercury into pores, against the resistance of liquid surface tension, is indicative of the pore size, assuming the pores are cylindrical in shape. However, the resolution of the technique is limited in scaffolds with large pore sizes ($>500\ \mu\text{m}$) where low mercury intrusion pressures are necessary, and it has limitations when applied to materials that have irregular pore geometries. Alternative techniques such as μCT , which utilize 3D CT imaging to generate computer models of porous materials, have become the modern standard for analyzing bone architecture and scaffold morphology. Using 3D μCT techniques, considerably greater information can be obtained to characterize pore architectures containing features ranging from 6 to $>1500\ \mu\text{m}$, without the physical limitations associated with mercury porosimetry.¹⁴

11.3.5 General scholium to scaffold design

Scaffolds designed for the regeneration of critically sized and/or of large volume defects require a clear vascularization strategy either based on the morphological design or a combination of morphology and biological derived facilitators (peptides, growth factors, cells, etc.). There are exceptions to this

statement; for instance, in the cornea or cartilage which does not have a vasculature. Vascularization is such a key issue in TE, that there is a separate chapter (see [Chapter 14 Vascularization, survival, and functionality of tissue-engineered constructs](#)) in this textbook dedicated to this topic.

11.4 Classical scaffold fabrication techniques

There are many different methods developed in the 1990 and 2000s to fabricate porous scaffolds. However, many of these techniques did not yet make significant impact and/or have no relevance for bench-to-bedside translation. The following scaffold fabrication approaches have been used for decades and, while still having utility, are subjected to less research than additive manufacturing techniques described in a future section.

11.4.1 Porogen leaching

Porogen leaching ([Fig. 11.2a and b](#)) was used in the early days of TE as it is one of the oldest polymer processing technologies to make porous products. It is based on dispersing a template (particles, etc.) within a polymeric or monomeric solution, gelling or fixing the structure, and removal of the template to result in a porous morphology. Recent improvements involved the combining shaking of regular sized spherical porogens, to produce extremely well-defined scaffolds⁶ that could positively alter the inflammatory reaction to the scaffold. Porogen leaching is applicable to various polymers and hydrogels,¹⁵ and dissolving out “sacrificial structures” ([Fig. 11.2c](#)) is a common occurrence in many fabrication approaches for TE, especially for vascularization¹⁶ or the guidance of ingrowing tissue.

11.4.2 Phase separation

Phase separation has been employed for decades for producing hollow fiber membranes and porous structures ([Fig. 11.2d](#)).¹⁷ Thermally induced phase separation (TIPS), in particular, has produced a range of scaffolds and is based upon the reduction of polymer solubility when the temperature is lowered, or when the polymer is frozen out of solution ([Fig. 11.2e](#)). These two types of TIPS are termed liquid–liquid and solid–liquid phase separation, respectively. Hydrogel scaffolds, described as “sponges,” can also be prepared by polymerization-induced phase separation.

11.4.3 Ice templating

The freezing of hydrogels has been used on many occasions to induce channels and pores ([Fig. 11.2f](#)). Various hydrogels have been transformed into scaffolds by freezing including agarose, cross-linked poly(ethylene glycol),¹⁸ chitosan,¹⁹ and collagen.⁸ Channels in the hydrogels reflect the ice crystal structures formed and can guide cell and/or tissue growth in the scaffolds. The size and scale of the pores is controlled by the temperature gradient, with larger ice crystals producing larger pores. They have been investigated primarily for tissue such as the peripheral nerve, spinal cord, and muscle repair.¹⁹

11.4.4 Micromolding

Despite the recent interest in additive manufacturing, the molding of porous objects remains relevant, particularly for higher production rates. Traditionally, once an object has been decided upon using rapid prototyping, molding offers an attractive approach to produce tens of thousands of such samples.

An excellent in vitro study on microvessels used micromolding to produce high fidelity structures for understanding blood vessel formation.²⁰ Another approach is to use additive manufacturing techniques to fabricate a complex structure that is then part of a micromolded structure.^{16,21}

11.4.5 Gas foaming

Gas foaming of biodegradable polymers found its original application in the biomedical sciences in drug delivery applications during the 1980s. It is a scaffold fabrication technique that permits solvent-free formation of porous materials through generation of gas bubbles within a polymer. Molded polymers may be pressurized with a gas, typically CO₂, until the polymer is saturated. The release of pressure results in nucleation and growth of the air bubbles up to 100 μm; however, interconnectivity is still limited and is often combined with **particulate leaching** to obtain improved interconnectivity between pores.

11.4.6 Classical nonwoven textiles

Cell-invasive fiber-based scaffolds can be produced using methodologies developed for the textile industry (Fig. 11.2e). TE textiles have a relatively high surface area and their “value-added” application, from an industry with established techniques, is an advantage. Additionally, textiles are typically formed into sheets and the permeability is high, allowing the necessary nutrients to reach the seeded cells. Many of the recent advances in fiber-based technologies have involved hydrogel fibers²² and “living fiber” systems. The latter involves the fibers being used as a carrier for cells.²³

11.4.7 Knitted and braided textiles

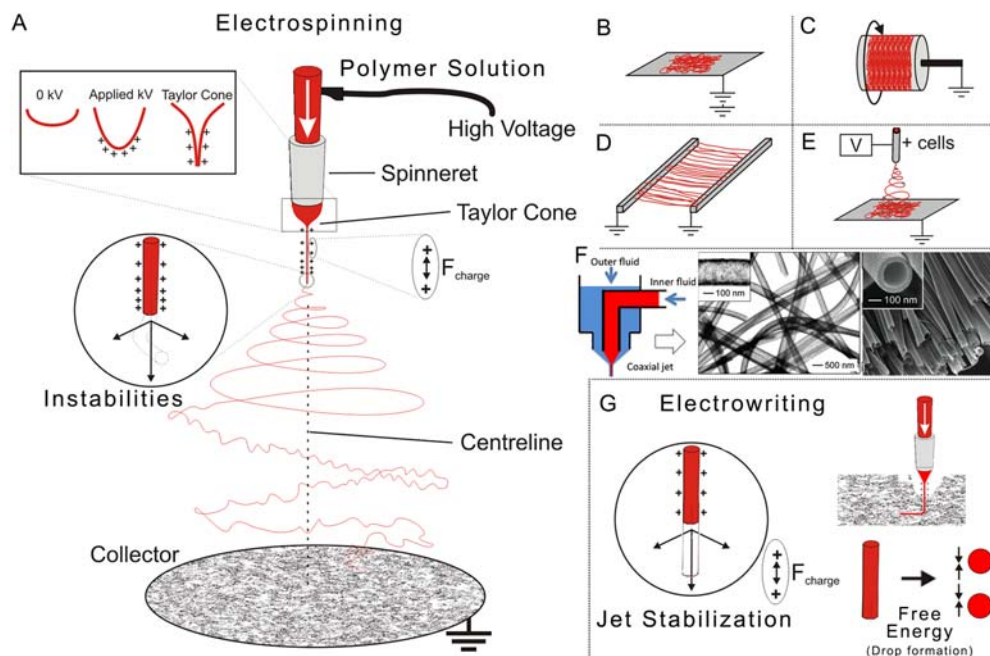
Individual fibers, or multifilament yarns, may be woven, knitted, or braided into patterns with variable pore sizes. Extremely complex fabric structures can result, and the relatively large pores between the fibers can be filled with a secondary scaffold, such as collagen gel or electrospun fibers (Fig. 11.2e).⁷ The fabric structure is, in this instance, a mechanical support for a secondary, interstitial scaffold that may be otherwise easily damaged. These approaches are often ideal for forming scaffold sheets or flexible rope-like scaffolds with excellent tensile properties for skin and ligament repair but not necessarily applicable to forming thick scaffolds used in application for **compressive loading**.

11.5 Electrospinning

Electrospinning is an inexpensive manufacturing technique for submicron and micron diameter fibers from polymer solutions (Fig. 11.2g) or melts (Fig. 11.2h). Although it is a process known since the 1930s, the past 2 decades have seen dramatic expansion of its use in TE applications, requiring specific attention in this chapter. Electrospinning has been widely investigated for scaffold fabrication; however, with hindsight, the technology lends itself more to fabricating membranes (Fig. 11.2i).

11.5.1 Electrospinning principles

A high (positive or negative) voltage is applied to a polymer solution or melt that is pumped to a spinneret (small orifice, or flat-tipped needle) facing an earthed target, or collector (Fig. 11.3). Upon

**FIGURE 11.3**

(a) Graphical illustration of the electrospinning process by using different collectors and nozzles. Common collector configurations for electrospun fibers (red) are (b) single ground, (c) rotating single ground, (d) dual bar, and (e) electrospinning cells within the polymer solution. (f) electrospinning with a coaxial nozzle, to form a compound Taylor cone and hollow fibers after center polymer is dissolved, (g) schematic showing how electrowriting operates with lower electric fields and is intended to prevent fluid breakup due to Raleigh instabilities. (f) From Li D, Xia Y. Direct fabrication of composite and ceramic hollow nanofibers by electrospinning. *Nano Lett* 2004;4(5): 933–938.

reaching a critical voltage, the surface tension of the polymer at spinneret tip is counterbalanced by localized charges generated by the electrostatic force, and the droplet elongates and stretches into a (Taylor) cone where a continuous jet is ejected. Initially, the polymer jet travels straight directly toward the target.

The surface charge density on this straight polymer jet increases closer to the collector (Fig. 11.3a). At a point often referred to as where “whipping” instabilities commence, the surface charges repel each other and result in spiraling and further stretching. Solvent evaporation or cooling of the polymer prior to landing at the collector results in electrospun fibers with sizes typically between 200 nm and 5 microns, depending on the conditions.

11.5.1.1 Collection systems

The collection configuration can greatly affect the collected electrospun material; however, most collectors are either flat (Fig. 11.3b) or a drum (Fig. 11.3c). Oriented fibers can be collected between grounded elements (Fig. 11.3d) and are suspended in air between the two grounds. An inverted

hemisphere containing wire filaments has also been used as a collector to increase the porosity of the electrospun material,²⁴ whereas collecting onto ice crystals while they are growing is another method to increase porosity.²⁵

11.5.1.2 Electric field variations

The electric field is what drives the polymer jet to the collector(s), and variations affect the deposition pattern of the electrospun fibers. The gap method of alignment has electric field force vectors separating close to the collector. As the fibers deposit from one collector to another, suspended oriented fibers result across the gap. The electric field is related to the collector shape; however, external manipulations are possible without changing the collector. Auxiliary electrodes used at a point below the nozzle²⁶ are able to rapidly direct the deposition of each fiber. Cells can also be added to the electrospinning media (Fig. 11.3e).²⁷ A significant proportion of advances within electrospinning have been achieved with new collector concepts.

11.5.1.3 Spinneret configurations

The **spinneret** can be a metal or an insulator and can potentially have a variety of shapes—including cylinders that rotate through a solution and electrospin off the cylinder surface. Slit and line spinnerets have also been used with success to produce multiple Taylor cones. Dual spinnerets have been used to form tubular constructs with different fiber types, including bimodal scaffolds.²⁸ Spinnerets can also be designed to deliver multiple fluids to within the Taylor cone. This approach is called **coaxial electrospinning** and it results in hollow electrospun fibers and can contain multiple channels within the fiber (Fig. 11.3f).

11.5.2 Cell/electrospun scaffold interactions

While initially proposed as a scaffold technology, nonwoven electrospun materials have limited cell penetration due to fiber diameters resulting in small pores (Fig. 11.2i). However, cell adhesion is excellent, when compared to flat surfaces. Many cells extend and orient along the electrospun fibers, while fibroblasts are particularly motile and stretch their cell body into “spindle-like” morphologies. The surface properties of electrospun fibers are important for cell adhesion and spreading and can be achieved either postprocessing, using chemical or physical adsorption, or during processing.

One shortcoming of solution electrospinning is that the very small fibers produced are often not conducive for cell penetration.²⁴ The lack of pore size and interconnectivity resulting from the chaotic deposition of electrospun fibers creates a barrier to cell invasion. Important fundamental research by Ref. 9 investigated the pore size generated when random fibers of different diameters are deposited upon each other. For random fibers, a critical fiber diameter of 4 μm was necessary for pores of 20 μm and higher.⁹ Therefore, approaches to expand the pores of electrospun fibers have been developed over the past decade,^{24,25} although issues pertaining to handling arise in many of these situations. Electrowriting, where lower charging conditions are used only to prevent droplet formation (Fig. 11.3g), has been developed to address the pore size issues and will be described later in this chapter.

11.5.3 Melt electrospinning

While solution electrospinning is widely researched, a fraction of melt electrospinning approaches has been reported to date. Electrospinning polymers without solvents (via the melt) may be attractive for applications and/or closed systems where solvent accumulation or toxicity is a concern. With a lack of solvents, melt electrospinning can be performed without a ventilation system, and the membranes (Fig. 11.2h) can be used immediately without further degassing. Melt electrospinning was originally considered as only making large micron-sized diameters; however, fibers as small as 270 nm have been reported.

11.6 Additive manufacturing

Additive manufacturing is the term used by industry, yet it is often portrayed as “3D printing” by the popular media; the latter term has become so widespread, that both terms have become interchangeable with each other in both the scientific literature and lay man journalism as well as the social media. **Rapid prototyping** refers to how additive manufacturing was initially used, to make prototypes that could then be scaled using conventional subtractive or formative manufacturing techniques. The recent advancements in additive manufacturing allow a final product to be made in a cost-effective manner.

Additive manufacturing technologies in TE^{29,30} can be broadly classified into three different approaches—(1) photorein-based, (2) nozzle-based, and (3) powder-based.³¹ Almost all selectively add material, layer-by-layer, as controlled by a computer program. There are recent volumetric fabrication approaches that use holographic approaches that are much faster without layer-by-layer delays.³² At the digital heart of additive manufacturing is the computer-aided design (CAD) model that is used to guide the 3D printer (Fig. 11.4). One benefit offered by additive manufacturing technology is the ability to create parts with compositional variation across the entire structure due to the computer-controlled nature of fabrication. Another strength of additive manufacturing is that the scaffold morphology can be discretely and systematically controlled.

In subtractive manufacturing (Fig. 11.4a), a block of material is processed by material-removing machines according to digital design before obtaining the final 3D object together with a large amount of residual material. From a scaffold perspective, subtractive manufacturing includes porogen leaching as the particles are subtracted from the original, overall shape. In additive manufacturing (Fig. 11.4b), a starting material (powder, liquid, filament, etc.) is processed by a 3D printer, which deposits just the required amount of material in a layer-by-layer approach to fabricate the object. The amount of residual material left over after additive manufacturing is significantly lower than that resulting from subtractive manufacturing. Fig. 11.4c shows how the digital surface tessellation language (STL) file is obtained using either CAD software, a 3D scanner, the internet, or using medical imaging. A slicing process (Fig. 11.4c) is then applied to the STL file using the printer software to convert this data into a G-code file containing geometrical information of a series of two-dimensional layers. Finally, the printer starts depositing the material following the layer-by-layer sequence dictated by the G-code file until the designed scaffold is fabricated.

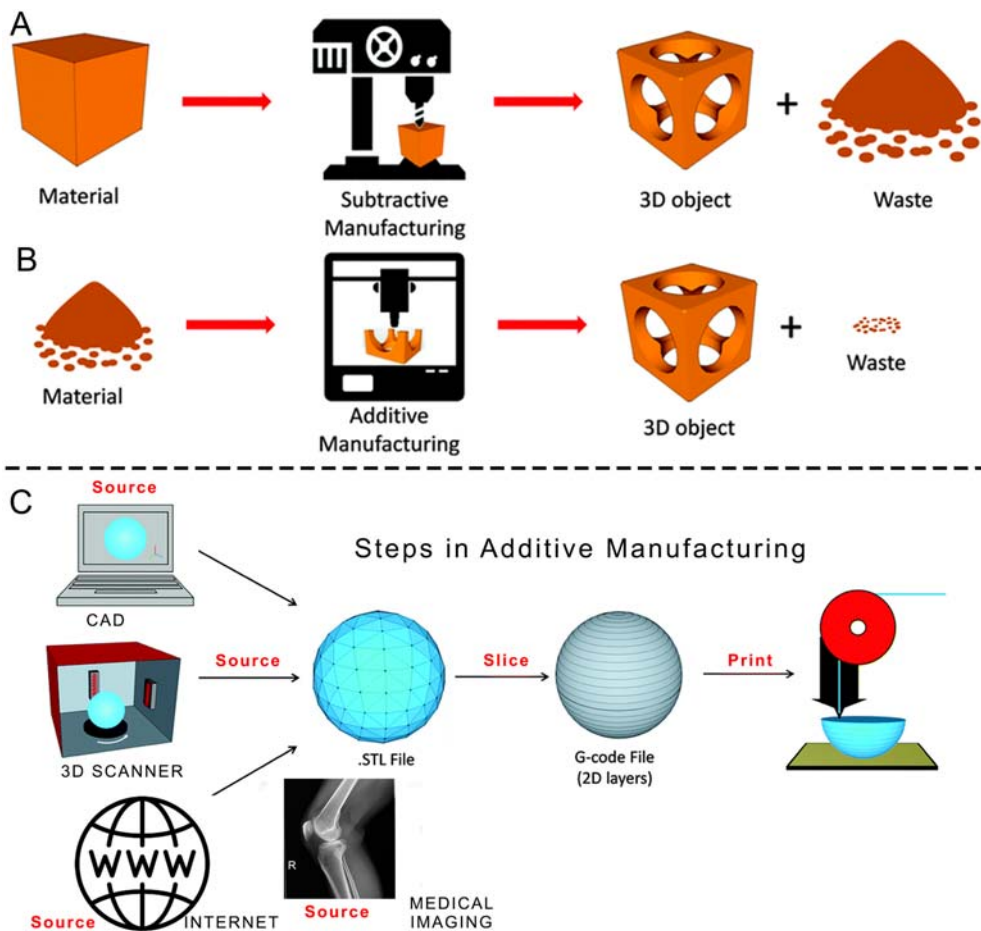


FIGURE 11.4

Schematics of demonstrating the differences between (a) subtractive manufacturing and (b) additive manufacturing. (c) shows a workflow of many additive manufacturing processes, using a surface tessellation language (STL) file created from different origins. *Adapted from Ambrosi A, Pumera M. 3D-printing technologies for electrochemical applications. Chem Soc Rev 2016;45(10):2740–2755.*

11.6.1 Direct writing and extrusion of polymers

Melt extrusion of strands/filaments has been developed more recently toward establishment as additive manufacturing technology. A typical machine consists of a heated liquefier head attached to a carriage moving in the horizontal x–y plane. Polymer is forced through the liquefier/heating zone to a nozzle to fabricate the scaffold following a programmed path which is based on a CAD model and the slice parameters (Fig. 11.4c). Once a layer is built, the platform moves down one step in the z-direction to deposit the next layer. Parts are fabricated layer-by-layer with the layer thickness varying in proportion to the nozzle diameter chosen. This high temperature process is restricted to the use of thermoplastic materials with good melt viscosity properties; cells or other

thermo-sensitive biological agents cannot be encapsulated into the scaffold during the fabrication process.

A design limitation of melt extrusion systems is that the pore size for the scaffolds may not be consistent in all three dimensions—as the melt requires a surface to be deposited upon, there are challenges with overhanging structures. The pores in both the x- and y-directions are formed in between the intersection of material strands/filaments and are determined by user-defined settings for fiber spacing. However, the pore size in the z-direction is formed from voids created by the stacking of material layers (Fig. 11.5a), and hence, their sizes are restricted to the thickness (diameter) of the deposited polymer fibers. Channels within hydrogels have also been made using **sacrificial fiber templates** (Fig. 11.5b), to study vascularization processes within matrices.

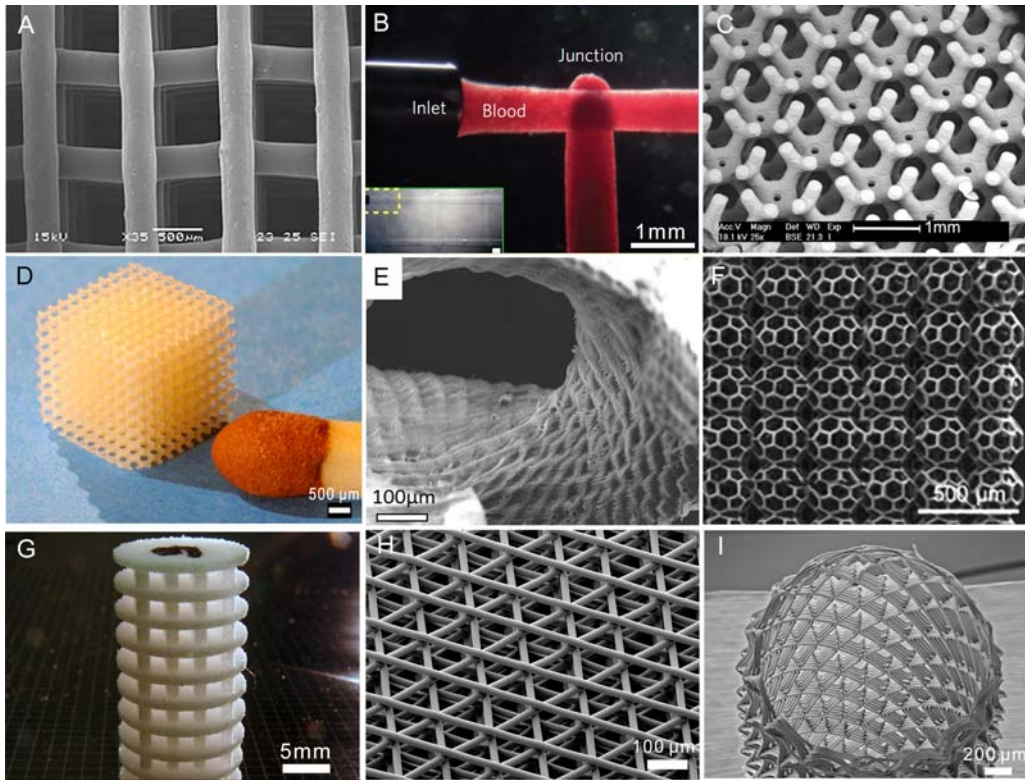
One technical limitation of mechanically extruding polymer melts is the large diameter of fibers produced: typically above 100 μm . The addition of a solvent to the polymer will permit smaller nozzle use, and therefore smaller filaments. However, the fidelity of the filament requires rapid gelling after passing through the nozzle. UV-cross-linking is an often used approach, and this has been adopted to cross-link polymer solutions³³ or cell-bioink formulations in a direct writing capacity.

11.6.2 Inkjet/powder systems (3D printing)

While 3D printing is today a generic term to describe additive manufacturing, that term was used to describe a specific technology developed in the 1990s at the Massachusetts Institute of Technology (MIT). It involved creating a solid object by selective ink-jet printing into a binder and repeating this in sequential layers. Each layer is created by spreading a thin layer of powder over the previous printed layer. The powder bed is supported by a piston which descends upon the powder spreading and printing of each layer (or, conversely, the ink jets and spreader are raised after printing of each layer and the bed remains stationary). The powder approach means that supporting structures are often not required. After the binder has dried in the powder bed, the finished component can be retrieved, and unbound powder recycled. The versatility of using a powdered material is both an advantage and a constraint of the inkjet/powder process. Many biomaterials do not come in a powder form and need special processing conditions to get a powder which fulfills the requirements for printing.

11.6.3 Stereolithography

Stereolithography (SLA) is often considered the pioneer of the additive manufacturing industry with the first commercial system introduced in 1988 by 3D Systems Inc. SLA is based on the use of a focused UV laser which is vector scanned over the top of a liquid bath of a photopolymerizable material. The UV laser causes the bath to polymerize where the laser beam strikes the surface of the bath, resulting in the creation of a first solid plastic layer at and just below the surface. The solid layer is then lowered into the bath and the laser generated polymerization process is repeated for the generation of the next layer, and so on, until a plurality of superimposed layers forming the desired scaffold morphology is obtained. The most recently created layer in each case is always lowered to a position for the creation of the next layer slightly below the surface of the liquid bath. Once the scaffold is complete, the platform rises out of the vat and the excess resin is drained. The scaffold (Fig. 11.5c and d) is then removed from the platform, excess resin washed off, and then placed in a UV oven for a final **curing**.

**FIGURE 11.5**

Additively manufactured scaffolds using (a) FDM, including (b) as a sacrificial template, Examples of SLA scaffolds at (c) high and (d) low magnification. (e) shows the morphology of a scaffold made using DLP. (f) Scaffolds made using 2PP, (g) SLS and (h) melt electrospun scaffold, as well as a (i) melt electrospun tube. (b) From Miller JS, Stevens KR, Yang MT, et al. Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nat Mater* 2012;11(9):768–774. (c) From Stampfl J, Baudis S, Heller C, et al. Photopolymers with tunable mechanical properties processed by laser-based high-resolution stereolithography. *J Micromech Microeng* 2008;18(12):125014. (d) From Melchels FPW, Feijen J, Grijpma DW. A poly(d,l-lactide) resin for the preparation of tissue engineering scaffolds by stereolithography. *Biomaterials* 2009;30(23):3801–3809. (e) From Lim KS, Levato R, Costa PF, et al. Bio-resin for high resolution lithography-based biofabrication of complex cell-laden constructs. *Biofabrication* 2018;10(3):034101. (f) Weisgrab G, Guillaume O, Guo Z, et al. 3D printing of large-scale and highly porous biodegradable tissue engineering scaffolds from poly(trimethylene-carbonate) using two-photon-polymerization. *Biofabrication* 2020;12(4):045036. (g) From Eshraghi S, Das S. Mechanical and microstructural properties of polycaprolactone scaffolds with one-dimensional, two-dimensional, and three-dimensional orthogonally oriented porous architectures produced by selective laser sintering. *Acta Biomater* 2010;6(7):2467–2476. (h) From Youssef A, Hrynevich A, Fladeland L, et al. The impact of mels electrospun scpfold dedign on porosityrdetermined by X-Ray microtomography. *Tissue Eng C Methods* 2019;25(6):367–379. (i) From McColl E, Groll J, Jungst T, Dalton PD. Design and fabrication of melt electrospun tubes using intuitive software. *Mater Des* 2018;155:46–58.

11.6.4 Digital light processing

3D printing systems based on digital light processing (DLP) involve use of light projection technology, developed by Texas Instruments in the 1980s, to polymerize materials for fabricating structures (Fig. 11.5e) in a layer-by-layer approach. The material being used should have adequate photosensitive properties for the process to function successfully. The light projection is controlled by a digital micro-mirror device which has micron-sized controllable mirrors to direct the path of light. DLP printing has a micron scale resolution, and the printing process occurs without high temperature, pressure, or shear forces enabling mild printing conditions for cells. DLP has the advantage of printing entire layer as opposed to a spot-by-spot approach opted in SLA enabling faster printing. The 3D-printed parts, similar to SLA, require postprocessing for support removal and curing/cross-linking the polymer completely. DLP has been used to make sacrificial structures that also can be embedded within a hydrogel.³⁴

11.6.5 Digital light synthesis

Digital light synthesis (DLS), also known as Continuous Liquid Interface Production (CLIP), is an additive manufacturing technology based on DLP but with a continuous layering approach. It is a photo-chemical process in which a series of UV images are continuously projected through an oxygen permeable membrane window on a liquid resin. The concept of continuously projecting the light through an oxygen permeable membrane is what makes DLS unique and differentiates it from DLP. This membrane creates a thin layer of liquid resin at the interface of the window and the printing part preventing the part from sticking to the window enabling easy postprocessing of the part fabricated. Another unique feature of DLS is the use of heat to cure the parts to enhance the mechanical properties.

11.6.6 Two-photon polymerization

Two-photon polymerization (2PP) produces results the best resolved additively manufactured products,^{35,36} with nano-scale tolerances, and the ability to produce very small pores and highly detailed structures (Fig. 11.5f).³⁷ Similar to that of SLA, a photo-curable resin is required, and currently only small objects can be produced. 3D photografting can produce functionalized regions within the hydrogel, while the opposite use of two-photon polymerization to weaken regions of a hydrogel matrix allows the control of cell migration.

11.6.7 Selective laser sintering

Selective laser sintering (SLS) also uses a focused laser beam, but to sinter areas of a loosely compacted powder.³⁸ This method begins with a thin layer of powder spread evenly onto a flat surface with a roller mechanism and then **raster-scanned** with a high-power laser beam. The powder material that is struck by the laser beam is fused, while the other areas of powder remain dissociated. Successive layers of powder are deposited and raster-scanned, one on top of another, until an entire part is complete (Fig. 11.5g). Each layer is sintered deeply enough to bond it to the preceding layer.

11.6.8 Melt electrowriting

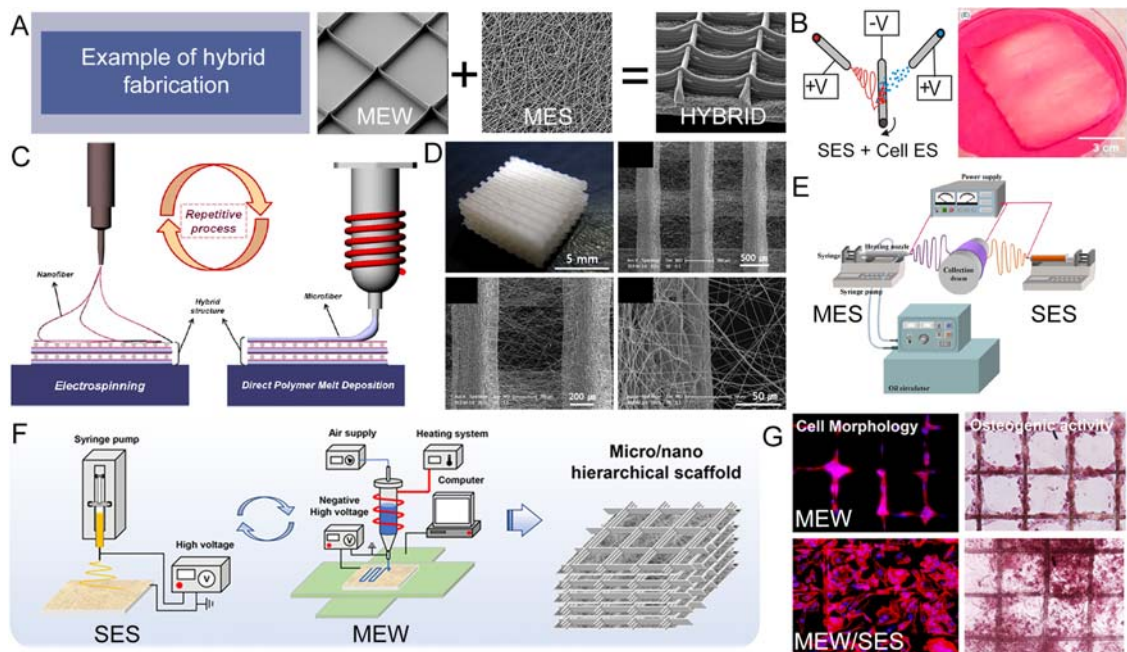
A hybrid form between melt extrusion and electrospinning is the use of a voltage to maintain a continuous, thin, jet onto a collector. In this way, melt electrowriting (MEW) has no “whipping” compared to melt electrospinning and is more conducive to rapid solidification and better printing resolutions than its polymer solution counterpart or solution electrospinning. The gold standard for MEW is medical-grade poly(ϵ -caprolactone) (PCL). Below a particular collector speed, the MEW jet will buckle and mechanically deform as a liquid. Above this speed, a straight fiber, typically 5–50 μm , can be direct-written into 3D shapes and structures that are normally porous and have a high surface area and volume (Fig. 11.5h). The thickest MEW scaffolds made to date are 7-mm thick and consist of 300 fiber layers. The fiber collection can also be performed on a mandrel collector to result in well-defined tube “scaffolds” (Fig. 11.5i), which were the basis of *in vivo* models to investigate cancer metastasis.³⁹

11.7 Hybrid fabrication

While we have introduced concepts of various scaffolds manufacturing processes in this chapter, in reality, different approaches are becoming increasingly combined. A common trend is to combine manufacturing approaches so that the benefits of each are provided while minimizing their disadvantages.⁴⁰ As an example of hybrid fabrication, when electrospinning does not produce sufficient porosities, then it can be combined with MEW to provide the volume and mechanical properties (Fig. 11.6a). One of the first hybrid fabrication approaches in TE involved combining solution electrospinning with cell electrospraying to form a construct (Fig. 11.6b). With solution electrospinning performed briefly during each scaffold layer made by melt extrusion, these scaffolds are termed multimodal, and commonly include submicron elements. Shown in Fig. 11.6c, solution electrospun scaffolds have low porosity; however, the submicron scale fibers are known to provide some important signals to cells, such as for adhesion and differentiation.^{41,42} Multimodal scaffolds combine these submicron solution electrospun fibers with micron-scale scaffold manufacturing approaches such as direct writing (Figs. 11.6c and d)⁴³ and melt electrospinning (Fig. 11.6e).²⁸ This results in scaffolds with high surface area due to the submicron fibers (promoting cell attachment and assisting in higher seeding of the scaffold), as well as a suitable porosity and pore size due to the microscale elements. Another example shows how solution electrospinning and MEW can be combined (Fig. 11.6f) to improve cell seeding of the scaffold and subsequent osteogenic activity (Fig. 11.6g). There are many more possibilities for generating multimodal scaffolds, and undoubtedly their fabrication will be the focus of future research.

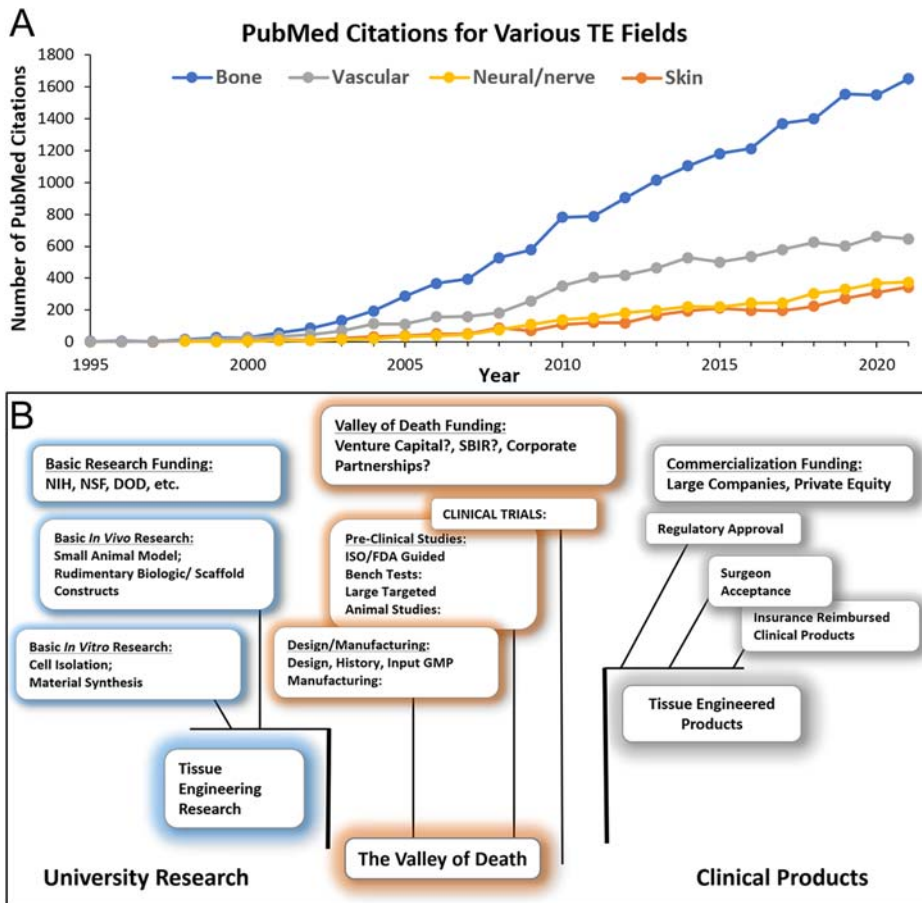
11.8 Clinical translation of scaffold guided tissue engineering

The field of SGTE was initiated nearly 5 decades ago to develop alternative treatment options that will ideally eliminate the previously described issues of current clinically used treatment concepts. The development and study of scaffolds has seen tremendous growth over the years, with an exponentially increasing number of studies and reviews published in the PubMed database. Fig. 11.7a shows the sustained increase in publications related to scaffold TE with similar trends found for bone, vascular, nerve/neural, skin TE, etc.

**FIGURE 11.6**

(a) Example of hybrid fabrication to combine different fabrication technologies. (b) combining cell electrospraying with solution electrospinning to form a cell sheet. (c) combining melt extrusion printing and electrospinning to design and fabricate (d) a multimodal scaffold architecture which has different scale elements. (e) shows how MES and SES can be combined to make larger and smaller diameter fibers together, respectively. (f) shows how SES and MEW are combined to build a multimodal morphology that (g) improves cell seeding and osteogenic activity. (a) From Dalton PD, et al. *Advances in Hybrid fabrication toward hierarchical tissue constructs*. *Adv Sci* 2020;7(11):1,902,953. (b) From Stankus JJ, Guan JJ, Fujimoto K, Wagner WR. *Microintegrating smooth muscle cells into a biodegradable, elastomeric fiber matrix*. *Biomaterials* 2006;27(5):735–744. (c, d) From Park SH, Kim TG, Kim HC, Yang DY, Park TG. *Development of dual scale scaffolds via direct polymer melt deposition and electrospinning for applications in tissue regeneration*. *Acta Biomater* 2008;4(5):1198–1207. (e) Kim SJ, Jang DH, Park WH, Min BM. *Fabrication and characterization of 3-dimensional PLGA nanofiber/microfiber composite scaffolds*. *Polymer* 2010;51(6):1320–1327. (f, g) Wang Z, Wang H, Xiong J, et al. *Fabrication and in vitro evaluation of PCL/gelatin hierarchical scaffolds based on melt electrospinning writing and solution electrospinning for bone regeneration*. *Mater Sci Eng C* 2021;128:112287.

In the context of clinical translation, the reader might ask the question “Is Scaffold-Guided Tissue Regeneration part of the field of TE or regenerative medicine (RM)?” In the literature, the terms TE and RM are often used interchangeably, though the specialists depending to which research community and/or society they belong (biomaterials, cell therapy, stem cell, genetic engineering, surgery societies, etc.) vehemently argue that they, in fact, represent different conceptual entities. For example, some groups argue though RM is a broader and more generalized field than TE, one does not wholly encompass the other. Both seek to restore function, but TE is narrower in its focus and does not require cellular regeneration. Nevertheless, taken together, one might argue that the two have grown to resemble a singular research entity. We argue in this book chapter that if the scaffold research is of fundamental nature

**FIGURE 11.7**

(a) Annual growth of studies on scaffolds for different fields of TE published in PubMed between 1995 and 2021. Search strategy: ((engineering) AND (*field*)) AND (scaffold). (b) The challenge of the “Valley of Death” is schematically shown, to highlight how this period between basic research and clinical products is an important one bound by time and funding. (a) *Is previously unpublished.* (b) *Adapted from Hollister S.J. Scaffold engineering: a bridge to where? Biofabrication 2009;1(1).*

it belongs to the TE field. If the scaffolds are researched in large preclinical animal studies and/or clinical trials the work should be defined as being part of the field of RM.

Unfortunately, what has not fundamentally changed, however, is the researcher/clinician interactions. It can even be argued that the TE&RM researcher and clinician relationship has deteriorated by looking at how many clinicians are members and/or attend flagship meetings of the societies. Based on successful clinical outcomes, the current path has inhibited rather than enhanced SGTE largescale bench-to bedside translation (Fig. 11.7b). Now, in the 21st century, some argue⁴⁴ that a traditional “scaffold” represents the a faulty approach, in that scaffolds that are designed to exactly replicate the niche, or

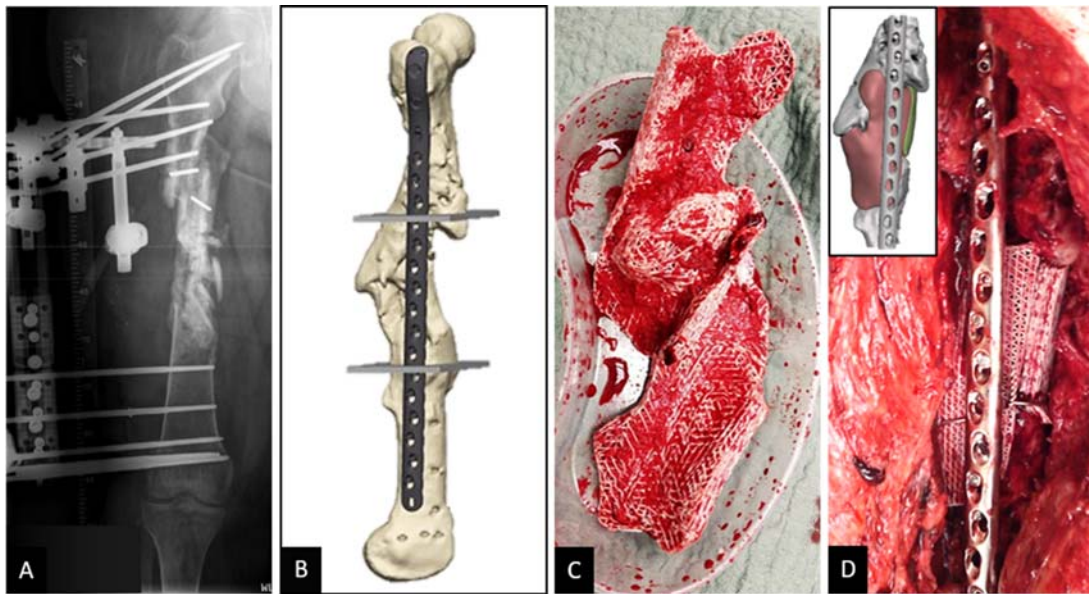


FIGURE 11.8

Treatment of large bone defect with complex malunion of the right femoral shaft with modular (*two parts*) 3D-printed PCL-TCP scaffolds. Following comprehensive treatment of a posttraumatic septic defect pseudarthrosis, including implantation of a hybrid fixator (*Orthofix*) to support stability during infect consolidation (a), 3D reconstruction of CT imaging data showed two distinguished femoral bone defects located antero-lateral and antero-medial and also the preferred plate osteosynthesis was integrated at an early stage in the surgical planning (b). Two geometrically matched 3D-printed PCL-TCP scaffolds loaded with autologous bone graft (c) were implanted and combined with plate osteosynthesis. Proper fit of the modular two-part scaffold of the bone defect as per the preoperative planning (see inset in d) was confirmed intraoperatively (d). *Fig. 11.8 previously unpublished.*

microenvironment, of these target cells are much likelier to succeed. Yet, replicating the niche exactly is only a hypothesis, since the microenvironment is constantly changing during the regeneration process and can take 1–3 years for complex large volume bone defects as shown in [Fig. 11.8](#) presented here. Furthermore, the research community frequently neglects the fact that there is often a great level of disengagement between the clinical demands of a TE concept and the scientific realization of the concept; a disconnect that hampers the clinical translation of the concept.⁴⁵ Although there is a clear and necessary role for discovery-based science in SGTE, there should be an increased focus on translational research and ultimately routine clinical application.⁴⁶

One of the first scaffold products was developed, clinically translated and commercialized by mechanical engineer Ioannis Yannas at the MIT in collaboration with burn surgeon John F. Burke at the Boston Shriners Hospital for Children and their colleagues.⁴⁷ It consists of a bovine type I collagen and shark chondroitin 6-sulfate mixture that is cross-linked and turned into a porous matrix by controlled freeze-drying. A silicone sheet attached to one side functions as a temporary epidermis-like barrier. Commercialized under the name Dermal Regeneration Template by Integra Life Sciences (Plainsboro, NJ), this product is used to cover severe burn wounds where the damage extends deep into the dermis. Under

these circumstances, the wound bed may not support a skin graft, or the absence of dermis may lead to extensive contraction and scarring of the healed wound. The matrix is biodegradable and presumably dissolves as the host's cells—primarily fibroblasts, endothelial cells, and neural cells—migrate into it and deposit their own ECM. Ultimately, the matrix disappears and is entirely replaced with a neodermis made of the patient's own cells and matrix, thus promoting dermal regeneration while inhibiting wound contraction and leading to better function and appearance of the healed wound. At that point, the silicone film is removed, and the wound is covered with a skin graft. Interestingly, the product contains no living cells, and its main purpose is to guide and stimulate the body's repair and regenerative processes.

These early successes fueled much enthusiasm, and many research laboratories embarked on applying TE to nearly every tissue in the body. Several new companies were spun off with great fanfare and the hope that, as some prominent spokespeople predicted in every decade, that tissue engineers would be able to not only develop but translate into routine clinical applications not only tissues but organs.⁴⁸ Some leaders in the field stressed over and over again that significant hurdles are to overcome and that these lab-based technologies have been developed largely by basic scientists and engineers, who have a tendency to oversimplify the problem and do not often recognize the clinical issues.⁴⁹ Nevertheless, some scaffold technologies have achieved a “bench to bedside” translation with large interdisciplinary teams that focused their energy on solving clinical problems to demonstrate the translation from SGTE^{50–52} (Fig. 11.9).

A reflection on the scaffold literature published over the last 5 decades of TE leads one to conclude that there is a gap between the bench and bedside. Acellular scaffolds deviate from this conclusion as they

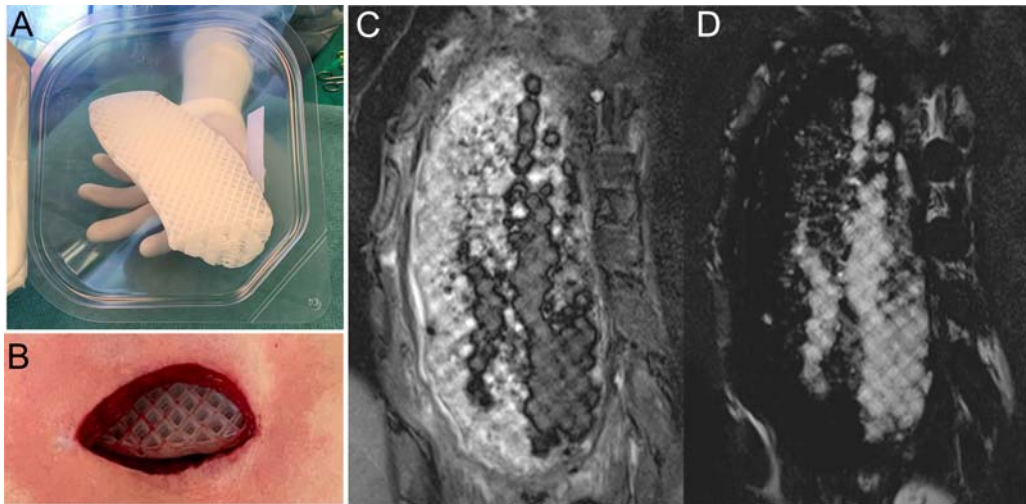


FIGURE 11.9

(a) A patient-specific pectus excavatum scaffold prior to implantation. (b) Intraoperative view shows an excellent fit of the pectoralis major scaffold inside the pocket. Magnetic resonance imaging of a fat-filled scaffold to correct a pectus excavatum deformity at 10 weeks using fat-suppressed (c) and water-suppressed (d) DIXON method. Adapted from Cheng ME, Janzekovic J, Theille HJ, et al. *Pectus excavatum camouflage: a new technique using a tissue engineered scaffold*. Eur J Plast Surg 2021.

are shown to perform as a template to guide different phases of the complex tissue regeneration triathlon, at a clinically relevant scale and in concordance with strict regulatory (economic and manufacturing) prerequisites. The scaffold should constitute a dynamically enduring yet degradable 3D architecture, preferably serving as a functional tissue substitute which, over time, can be replaced by cell-derived tissue function. Designing and manufacturing processes are the gatekeepers to translate TE research into clinical translation. One of the greatest difficulties in bridging the Valley of Death (Fig. 11.7b) is to develop, in research laboratories or small spin off companies, GMP and scalable designs and to apply these to preclinical studies. In order to take TE research from bench to bedside, it is also imperative to meticulously assess the clinical demands for specific scaffold characteristics to achieve a broad and optimized range of clinical applications for the specific TE approach.

11.9 Future perspective

Scaffolds are an important part of TE&RM since they enable to create a physical 3D environment that guide cells through the proliferation and maturation/remodeling phases including a patient's own cells harvested during surgery. In addition to considerations of scaffold performance, several forethoughts of how they are manufactured arise. For wider and routine clinical applications, scaffolds must be manufactured in a reproducible, controlled fashion at an economic cost and speed, and that meet necessary regulatory standards. The rise of additive manufacturing technologies has enabled this perspective, with automation additionally providing a surgical protocol rooted in a patient-specific implant.

It is a recent trend, driven by additive manufacturing, to produce scaffolds with well-defined morphology and interconnected porosity for many different cell types. As described previously, the scaffold has several biological requirements that include "(1) an immobilization site for transplanted cells and/or host cells, (2) formation of a protective space to prevent unwanted tissue growth into the wound bed and allow healing with differentiated tissue, (3) directing migration or growth of cells via surface properties of the scaffold, and (4) directing migration or growth of cells and ECM via release of soluble molecules such as growth factors, hormones, and/or cytokines." Scaffold manufacturing techniques must offer the right balance of capability versus practicality to be suitable for fabrication of materials in sufficient quantity and quality to allow holistic TE technology platforms to enter clinical application.

A key challenge of vascularization remains throughout the entire scaffold volume, while ensuring cells are distributed in the required locations and are sufficiently nourished. Furthermore, there exist three possibilities of incorporating cells into the scaffolds: (a) seeding of cells onto the scaffold surface subsequent to scaffold fabrication and (b) the incorporation of cells into the scaffold fabrication process or (c) implanting a cell-free scaffold directly into the body.

In summary, additive manufacturing is primed to have a far-reaching impact of scaffold-guided TE over the next decade. Several cell-free scaffolds for bone, skin, and trachea TE have been translated into routine clinical applications. However, the next decade should see advances in soft TE as a result of higher resolution additive manufacturing. The diversity in applications for scaffold use is set to increase, as in vitro models for injury and disease are refined and increasingly used by both the scientific and industrial communities.

Definition of tissue engineering and regenerative medicine

Defined at

<https://www.nibib.nih.gov/science-education/science-topics/tissue-engineering-and-regenerative-medicine>.

"Tissue engineering evolved from the field of biomaterials development and refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues. The goal of tissue engineering is to assemble functional constructs that restore, maintain, or improve damaged tissues or whole organs. Artificial skin and cartilage are examples of engineered tissues that

have been approved by the FDA; however, currently they have limited use in human patients."

"Regenerative medicine is a broad field that includes tissue engineering but also incorporates research on self-healing—where the body uses its own systems, sometimes with help foreign biological material to recreate cells and rebuild tissues and organs. The terms 'tissue engineering' and 'regenerative medicine' have become largely interchangeable, as the field hopes to focus on cures instead of treatments for complex, often chronic, diseases."

Summary

- To design a scaffold, its morphology, porosity, pore interconnectivity, pore characterization, and fabrication techniques should be considered.
- The most relevant scaffold fabrication techniques are porogen leaching, phase separation, ice templating, micromolding, gas foaming, nonwoven textile techniques, knitting, and braiding.
- Electrospinning is a flexible and promising scaffold fabrication technique. The cell/electrospun scaffold interactions should be thoroughly characterized.
- Additive manufacturing comprises direct writing and the extrusion of polymers, inkjet/powder 3D printing systems, SLA, DLP, SLS, and MEW.
- Despite all of these fabrication techniques, none of them can readily introduce a functional vascular network throughout a scaffold during the fabrication step.

Classical experiment

Collagen scaffolds

It is said that Joseph Vacanti was walking on the beach at Cape Cod in 1987 watching seaweed floating in the ocean and called to ask his friend Robert Langer to design and fabricate a device mimicking this branching structure for his liver research. Six years later, in a seminal 1993 paper, Langer and Vacanti proposed that "the scaffold should vanish away as the tissue is growing." Over the last 4 decades, thousands of research papers were based on this concept that led to minor translational research outcomes.

The early TE community neglected the importance of the "fourth dimension" or "time." Crucially, if the scaffold degrades before the tissue is matured and remodeled, the tissue regeneration is severely compromised. Hence, it is important to emphasize that both hypotheses, fast versus slow scaffold degradation, are rival empirical hypotheses to be settled by scientific research and not by philosophical argument. There are at least three lines of research that are consistent with, and often used to support the slow scaffold degradation theory.

Classical experiment—continued

Yannas and Burke proposed 5 decades ago that a highly porous implant should guide the tissue formation and its maturation and then start degrading.^{53,54} In 1976, Yannas and Burke might have been the first who defined the structural features of what we define today as a “scaffold.” The publication describes a highly porous analogue of the collagen ECM—hence what Yannas and Burke defined as a template was made of type I collagen—with highly specific structural features. These required features included

a specific range of the pore size, defined degradation half-life, and specified surface chemistry. They did not use the term “scaffold” yet refer to it as the “dermis regeneration template” as the research showed full-thickness skin wounds in animal and humans led to regeneration of a nearly physiological dermis (Fig. 11.10).

With receiving of FDA approval in 1996 for clinical use, freeze-dried collagen-GAG scaffolds are used to regenerate

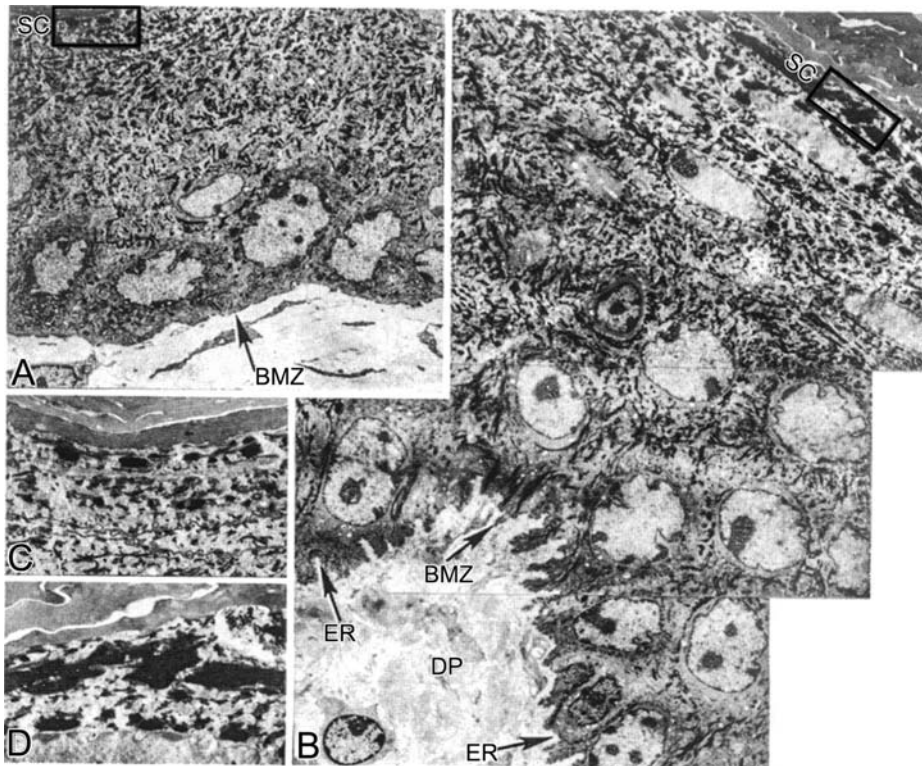


FIGURE 11.10

The ultrastructure of (a) full-thickness normal guinea pig epidermis and (b) the regenerated epidermis 1 year after grafting (identical magnifications). (b) shows that the epidermis is thicker and contains an increased number of nucleated cells than normal skin (a). (c) Comparing the keratohyaline granules (inset a) of the normal epidermis beneath the stratum corneum (SC) with the reconstituted epidermis, large and irregular contours are observed (d). DP, dermal papilla; BMZ, basement membrane zone; ERs, epidermal rete ridges (a and b, x 1200; c and d, x 2500). From Yannas IV, Lee E, Orgill DP, Skrabut EM, Murphy GF. *Synthesis and characterization of a model extracellular matrix that induces partial regeneration of adult mammalian skin*. Proc Natl Acad Sci USA 1989;86(3):933–937.

Continued

Classical experiment—continued

skin after burn injuries. With a pore size of 20–120 μm and 95% porosity, there is also a silicone coating on one side to reduce dehydration of the scaffold. This silicone-coated side is facing outward as it is placed on the injury site. A physiological dermis regenerates within the first 15 days while cells migrate into the scaffold in the first days/weeks. Epidermis from an uninjured

part of the skin is then removed, perforated, expanded to cover a larger area, and grafted onto the regenerated dermal bed. The wound is closed once epidermal tissue migrate and proliferate rapidly. It is also possible to form both epidermis and dermis simultaneously, when seeded with keratinocytes before implantation (Fig. 11.11).

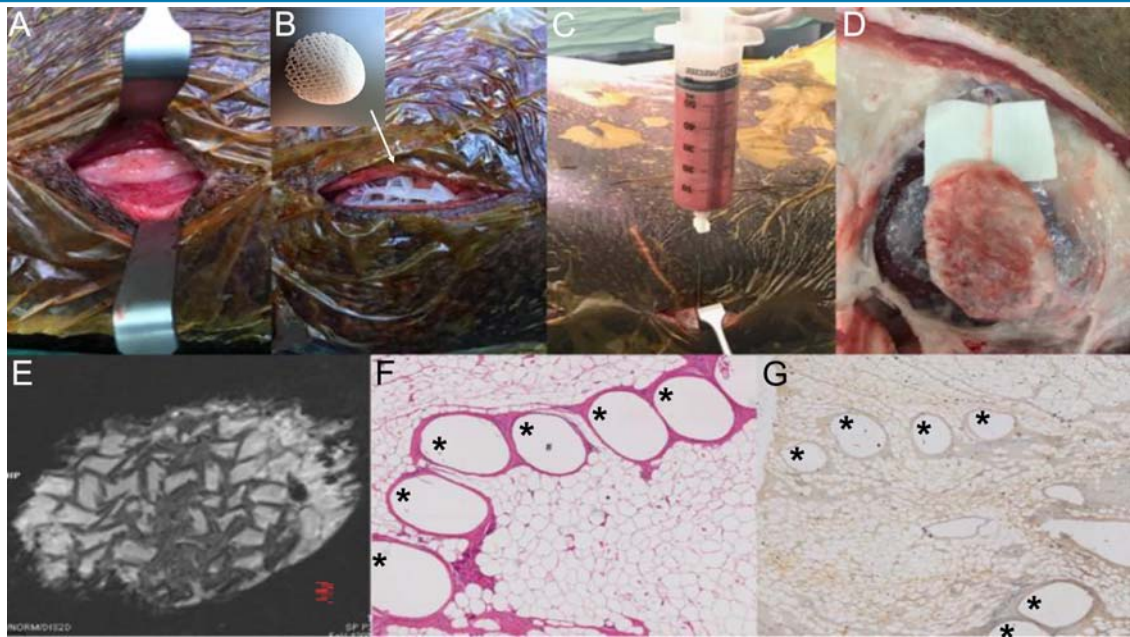


FIGURE 11.11

(a) A miniature pig model is used to implant (b) the PCL scaffold in situ. (c) An autologous fat graft is prepared for injection into the scaffold. (d) 12 months postimplantation the explanted scaffolds are vascularized and retain their original shape. (e) Scaffolds 1-year postimplantation. (f) Perilipin staining of tissue sections show healthy, viable adipocytes. (g) Staining tissue with hematoxylin and eosin (H&E) shows a predominance of adipose tissue supported by a fibrous tissue network surrounding scaffold struts (indicated with an asterisk). From Janzekovic J, Hunt J, Peltz T, Wagels M, Brown T, Hutmacher DW. Biomechanical Principles of Breast Implants and Current State of Research in Soft Tissue Engineering for Cosmetic Breast Augmentation. *Aesthetic Plastic Surgery*; 2021.

State-of-the-art experiment

Scaffold-guided breast reconstruction

Adipose TE has progressed significantly in the past decade and many research groups focus on small sized scaffolds which do not address the issue of large volume tissue defects. An important consideration during fabrication of scaffolds for large volume defects is the mechanical properties and integrity of the scaffold which depend on the biomaterial and morphology (e.g., porosity, pore size, and interconnections) of the scaffold. The designed highly porous implant should be able to withstand sustained regeneration over prolonged period of times and be designed in such a way that the transduction of external and physiological stresses is minimized to the newly forming tissue and allows for tissue maturation and remodeling (2–3 years). Mechanical stress is known to impede adipogenesis and is one of the major causes for compromised regeneration after autologous fat injection for breast reconstruction.

Recently, several research groups have focused on the additive manufacturing of medical grade PCL to fabricate implants for scaffold-guided breast reconstruction. The shape and internal structure of the scaffolds is as fundamental as the overall mechanical properties. The interconnected architecture of the scaffold guides and facilitates the formation of highly organized tissue with interconnected vasculature within the pore space of the scaffold.⁵⁵ A fully interconnected pore network is a *conditio sine qua non* for infiltration of cells within the scaffold as ECM begins to be laid out. Understanding the mechanism of ECM formation and remodeling is essential as it enables successful tissue regeneration instead of repair. It is important to optimize the correct balance of mechanical properties, morphology, and overall degradation of the scaffold to obtain a TEC which is routinely clinically applied.

Janzekovic et al., 2021, Biomechanical Principles of Breast Implants and Current State of Research in Soft Tissue Engineering for Cosmetic Breast Augmentation.

11.10 Recommended literature

1. Yannas IV. Tissue regeneration by use of collagen-glycosaminoglycan copolymers. *Clin. Mater.* 1992;9(3–4):179–187.
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5. Hollister SJ. Scaffold design and manufacturing: from concept to clinic. *Adv. Mater.* 2009;21(32–33):3330–3342.
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7. Dalton PD, et al. Advances in Hybrid fabrication toward hierarchical tissue constructs. *Adv. Sci.* 2020;7(11):1,902,953.
8. Kobbe et al. Convergence of scaffold-guided bone regeneration and RIA bone grafting for the treatment of a critical-sized bone defect of the femoral shaft. *Euro. J. Med. Res.* 2020;25(1):1–12.
9. Mieszczanek P, et al. Convergence of Machine vision and melt electrowriting. *Adv. Mater.* 2021;2,100,519.
10. Henkel J, et al. Scaffold-guided bone regeneration in large volume tibial segmental defects. *Bone* 2021;153:116,163.

11.11 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
1. What properties should you consider when designing your tissue engineering scaffold?
 2. What do you need to consider when choosing a degradable polymer for your scaffold?
 3. What are the four terms presented as the 4F concept?
 4. What is the relationship between molecular weight loss and mass loss of a hydrolytically degradable scaffold with time?
 5. What does nonantigenic, noncarcinogenic, nontoxic, and nonteratogenic mean with respect to tissue engineered constructs?
 6. What is nanoporosity with respect to scaffold design?
 7. Name four examples of manufacturing technologies that result in classical scaffolds.
 8. Name two measurement technologies that can allow you to quantify scaffold porosity.
 9. Draw a schematic of the main components required for solution electrospinning.
 10. What are the advantages and disadvantages of using melt electrospinning instead of solution electrospinning?
 11. How is melt electrowriting different from melt electrospinning?
 12. What is the difference between additive manufacturing and rapid prototyping?
 13. Name three ways that an STL file can be generated for additive manufacturing.
 14. Name five examples of additive manufacturing technologies for making scaffolds.
 15. What is the difference between DLP and DLS/CLIP?
 16. Name the additive manufacturing technology that results in the best resolved scaffolds.
 17. What is the gold standard polymer used for melt electrowriting?
 18. Name two types of materials that are used for powder-based 3D printing.
 19. Name two scaffold-guided regeneration strategies that have resulted in clinical implantation.
 20. What was one of the first scaffold technologies clinically translated?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:
1. What is the difference between a scaffold pore and scaffold porosity, and expand on the different types of pores that can be found?
 2. Describe the porosity and mechanical property relationship and how this can change with time.
 3. Describe how solution electrospinning works to result in small diameter fibers.
 4. How does the collector shape affect the types of fibers that are solution electrospun? Give examples.
 5. Describe the difference between subtractive manufacturing and additive manufacturing.
 6. Explain the steps in how an object is additively manufactured.
 7. Describe how digital light processing (DLP) works.
 8. Design and describe a hybrid manufacturing approach that combines different technologies.

9. Why has the field of tissue engineering and regenerative medicine been limited in the clinical translation of its products?
10. Describe the concept of the “Valley of Death,” with respect to clinical translation.

Challenge-based learning

In situ repair of large ventricular septum defects

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Embryologic development of the heart is complete at 8 weeks of gestation, but many congenital heart problems such as the ventricular septum defect (VSD) can arise. Surgery to correct major VSD is done in utero or after birth. However, there is a clinical need to develop minimally invasive surgery to introduce new materials to correct septal defects in the heart. The long-term vision is to develop noninvasive surgery to solve VSD with smart materials that can readily attach to the beating heart and withstand the high mechanical loads in the beating heart, without detrimental effects to the fetus in the womb.

Motivation and stakeholders

Heart septa's function is to divide atria and ventricles in two distinct chambers and prevent blood flow between them. Atria and ventricle septa consist of an internal myocardium layer sandwiched between two endocardium layers. Currently, large septal (wall) defects can only be solved using invasive surgery after birth but noninvasive, catheter-based techniques are being optimized to deliver biomaterials via major blood vessels to the heart. To strengthen these techniques, rapid prototyping is capable to produce custom-designed scaffolds for a diverse array of biomedical applications. Thus, the potential exists to design a new family of VSD scaffolds to successfully respond to the dynamic environment of the beating heart. Solutions to mitigate VSD should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as pediatric cardiac surgeons, neonatologists, pediatric nurses, material scientists, and biofabrication engineers.

Problem definition

Current rapid prototyping strategies mainly rely on a layer-by-layer approach to generate scaffolds. So far, no scaffolds have been fabricated to mimic the triple layer in native ventricle septum and with mechanical properties robust enough to withstand contraction and relaxation. Additionally, for VSD, the scaffold should be delivered non-invasively and with the capacity to tightly attach to the ventricle wall.

Challenge

To create a hybrid fabrication strategy that can generate adequate scaffolds for large VSD.

Learning framework

Reading the Scaffold Design and the Cardiovascular Tissue Engineering chapters will help you to understand the following:

1. The tissue architecture of the heart and especially of the septum.
2. Phenotype of the endo- and myocardial cells.
3. The tissue defects observed in VSD.
4. Current surgical approaches to treat VSD.
5. The mechanical compliance of scaffolds used in cardiovascular engineering.
6. The maintenance of endo- and myocardial phenotype of cells in contact with scaffolds.

For a more detailed look into your challenge, prepare a mind map summarizing the following:

7. Scaffold biofabrication techniques: from layer-by-layer manufacturing to volumetric additive manufacturing.
8. Biomaterials used for cardiac tissue engineering.
9. Mechanical requirements of biomaterials in cardiac tissue engineering.
10. Methods to deliver materials in situ.

Continued

Challenge-based learning—continued

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

11.12 Glossary

4F concept comprises the four essential components of a scaffold form, function, formation, and fixation.

Additive manufacturing is the construction of a three-dimensional object from a computer-assisted design model or a digital 3D model.

Bulk materials are those dry materials which are powdery, granular, or lumpy in nature, and are stored in heaps.

Coaxial electrospinning is a modification of electrospinning involving multiple feeding systems to simultaneously electrospin several polymer solutions.

Compressive loading Under compressive load, materials can only load up to a critical level after which bending deformation takes place. Buckling is considered as a critical property of many engineering materials and determines the failure criteria for a structure under high compressive load.

conditio sine qua non is an indispensable condition, action, or ingredient.

Curing is a chemical process that produces the toughening or hardening of a polymer material by cross-linking of polymer chains.

Electrospinning is a fiber production method that uses electric force to combine charged threads of polymer solutions to produce fibers in the order of hundreds of nanometers in diameter.

Mass loss is the loss of scaffold/polymer material due to degradation and erosion.

Particulate leaching is a technique used to create three-dimensional, porous scaffolds by mixing a polymer mixed with salt particles. The removal of salt results in a porous structure.

Phase separation is the creation of two distinct phases from a single homogeneous mixture.

Porogen-leaching is a common approach to developing large, three-dimensional, and porous scaffolds.

Power law relationship is a functional relationship between two quantities, where a relative change in one quantity results in a proportional relative change in the other quantity, independent of the initial size of those quantities. It can be mentioned that one quantity varies as the function of the power of another quantity.

Rapid prototyping is a group of techniques used to quickly fabricate a scale model of a physical part or assembly using three-dimensional data and computer-aided design.

Raster-scanned A raster scan, or raster scanning, is the rectangular pattern of image capture and reconstruction projected on a screen.

Regenerative medicine deals with the process of replacing, engineering, or regenerating human or animal cells, tissues, or organs to restore or establish normal function.

Sacrificial fiber templates are used to make scaffolds containing channels. The template is “sacrificed” or removed following polymer deposition on the template.

Spinneret is a device used to extrude a polymer solution to form fibers.

Tissue engineering is the design and fabrication of living replacement devices for surgical reconstruction and transplantation.

Tissue engineering construct is a biologically, functionally, and cosmetically successful replacement part.

Wound contraction is a physical deformity characterized by skin constriction and restrictions of functions during tissue recovery.

μCT is a technique that uses X-rays to create cross-sections of a physical object used to recreate a virtual model without destroying the original object.

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Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Controlled release strategies in tissue engineering

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12.1 Learning objectives

After reading this chapter you will be able to:

- Understand the methods employed in controlled release of biological factors.
- Gain an understanding of the clinical need for controlled release strategies of biological factors in tissue engineering.
- Understand the influence of the physicochemical properties of the incorporated bioactive agent on its controlled release.
- Be able to explain the classes of morphogens, list the ones important in tissue engineering, relate their biological function to therapeutic purpose, and the implications of their mode of action on appropriate controlled release strategies.
- Appreciate examples of current success and future challenges of controlled morphogen release in tissue engineering therapies.

In commenting on the remarkable passion with which tissue engineering scientists from different disciplines work together to develop controlled release strategies for tissue repair scaffolds, “Kids are dying,” Linda Griffith from MIT said, “so people tend to work together.”

Quoted in the New York Times, 1996.

12.2 Introduction

Controlled release of cell signaling molecules plays an integral role in the overall task of tissue engineering. As a result, tissue engineering strategies often depend heavily on delivery of bioactive signals, through bound or diffusible growth factor signals, or substrate-bound cell adhesion signals. Cells can be transplanted within such scaffolds and matrices; for example, cells have been cultured and placed there in vitro. In many cases, one must coax the cells to behave in desired ways by providing them with bioactive signals in a controlled manner. It is sometimes even hoped that, by providing bioactive signals within a matrix, we may succeed in coaxing cells, including progenitor and stem cells,

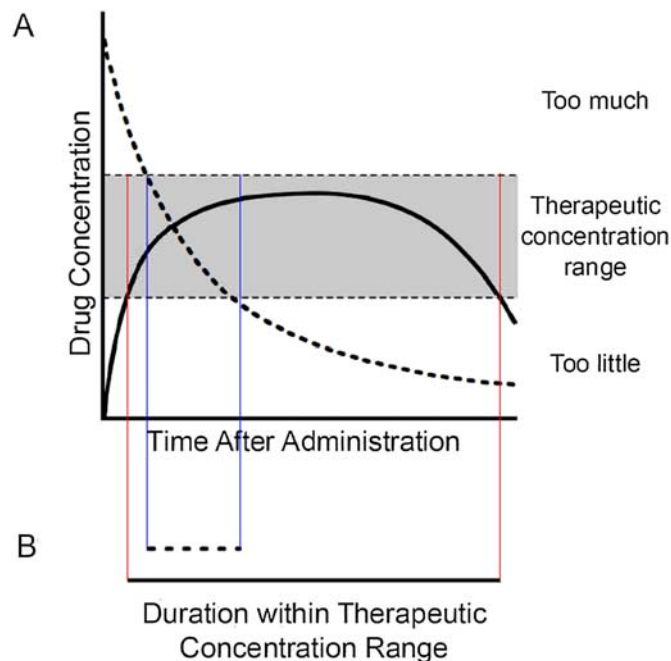
to migrate into the matrix from the surrounding tissues, to obviate the need for cell transplantation, and the logistical complexities that are associated with these. Thus, controlled release strategies are needed to provide signals to cells in the vicinity of the scaffolds and matrices and within the constructs to behave in desired ways.

In vitro, when cells are cultured within a scaffold or matrix, controlled release of biological factors may not be necessary: the bioactive signals may simply be added to the medium in the bioreactor. Controlled release may be beneficial, even if not necessary, to reduce the amounts of very expensive signals, such as growth factors, by providing them only locally within the scaffold or matrix. However, for in vivo applications, it makes little sense to administer such factors to the whole system. **Systemic** delivery could mean that very little and possibly even no growth factor would find its way to the implant, being cleared by other pathways in the body before it reaches that site. The systemic delivery also increases adverse side effects limiting the use of the needed **morphogens**, which are bioactive agents that help control morphogenesis. Thus, in vitro, controlled release may be beneficial, and in vivo it is usually essential.

The term *controlled release* is often taken to refer to release of a bioactive molecule from the bound state to a freely diffusible state, whereupon it can exert its biological activity. In tissue engineering, however, many of the bioactive molecules of interest are active in their immobilized state, and some, such as growth factors, can be active either immobilized or freely diffusible. As such, in this chapter, we shall use the term *controlled release* to refer to the provision of a bioactive molecule over time in a manner such that its biological activity can be productively harnessed. Thus, **controlled release** more precisely refers to the controlled provision of a biologically active molecule in its most appropriate state. For a molecule promoting cell adhesion, controlled release would refer to immobilization of that ligand and presentation on a surface for a prolonged duration. For a growth factor that signals intracellularly, it would refer to release of the factor into the diffusible state, so that it could be bound by the corresponding receptor and internalized.

The overall goal of the controlled release of signaling molecules is to present a biologically active factor to the tissues at a concentration that is optimal—not so low as to be ineffective and not so high as to display toxic side effects; this concentration range is known as the **therapeutic window** (gray box), as illustrated in Fig. 12.1. If simply injected into the tissue site as a bolus, without incorporation into a biomaterial scaffold or matrix, release will usually occur as a sudden burst, perhaps well above the upper limit of the therapeutic window, and then rapidly decline to concentrations that are too low to be effective, as depicted by the dashed line (Fig. 12.1A). If released slowly from a scaffold, a typical case in tissue engineering, the concentration of the drug in the vicinity of the implant would rise to within the therapeutic window, remain there for a prolonged duration while the desired signaling occurs, and then gradually decline to zero as the factor is depleted from the scaffold, as depicted by the solid line. Ideally, the drug is within the concentrations of the therapeutic window for the required amount of time during the healing process (Fig. 12.1B).

A number of strategies have been explored and can be envisioned for controlled release of biological signals in tissue engineering (Table 12.1). These are depicted in Fig. 12.2: (1) physically entrapping factors within gel networks, (2) entrapping factors within hydrophobic degradable polymers that either make up the matrix or that are used to form microparticles that are themselves entrapped within the

**FIGURE 12.1**

Engineering controlled release to increase therapeutic window.

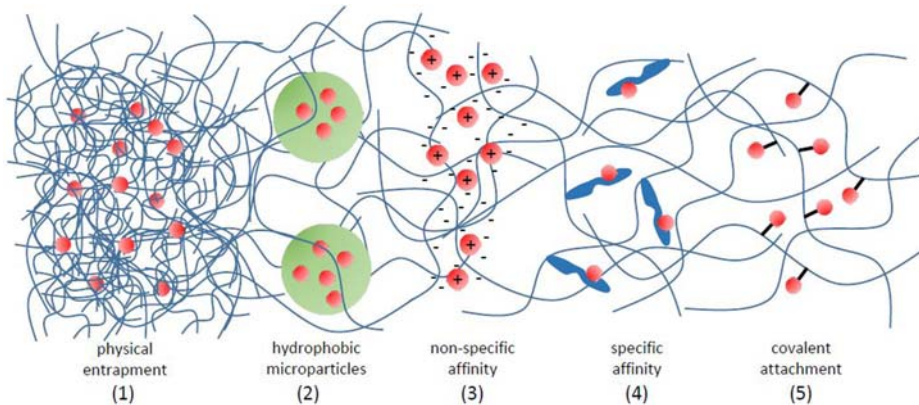
matrix, (3) binding factors within a matrix biomaterial that has nonspecific affinity for the factors, (4) binding factors to specific affinity sites naturally present in the matrix substrate or to affinity sites conjugated to the matrix (e.g., domains of fibronectin), (5) covalent binding of factors directly to the matrix material, and (6) engineering factors with an affinity to the endogenous matrix.

12.2.1 Bioactive molecules of interest in tissue engineering

Before considering the approaches to controlled release that are likely to be successful in tissue engineering, it is important to consider what types of molecules might be important to release. For example, release of low-molecular weight hydrophobic drugs, as might be used in contraception, inflammation reduction, or cancer, requires vastly different material types than might be used in the release of proteins drugs, as might be used in diabetes or growth hormone deficiency. In tissue engineering, it is these latter biomolecules that are of more interest. These proteins include growth factors, such as vascular endothelial growth factors (VEGFs), bone morphogenetic proteins (BMPs), platelet-derived growth factors (PDGFs), and interleukins (see Chapters 2–4 on the biological aspects of tissue engineering); adhesion factor morphogens, such as whole and domains of fibronectin, vitronectin, and laminin; and peptide mimics of these proteins. These molecules are large (molecular weight 5000–2,000,000 Da), are water soluble, and are very sensitive to their environment. Even small changes in their environment, such as exposure to hydrophobic surfaces, can result in protein denaturation and loss of biological activity. Perhaps even more importantly, the fraction of the protein that

Table 12.1 Drug delivery strategies encountered in tissue engineering.

Approach	Method of control	Example
Simple physical mixtures	None or physicochemical	Growth factors in collagen
Dense gel networks	Physical entrapment	Growth factors in synthetic polymer hydrogels
Polymer fibers and particles	Physical entrapment	Growth factors or small molecules in microparticles
Nonspecific affinity mixtures	Noncovalent electrostatic affinity	Growth factors in heparin containing matrices
Specific affinity mixtures	Noncovalent biochemical affinity	Growth factors in fibronectin containing matrices
Chemical immobilization	Covalent	Cell adhesion factors on scaffolds
Engineered morphogens with affinity to endogenous matrix	Noncovalent engineered biochemical affinity	Engineered growth factors with matrix binding domain

**FIGURE 12.2**

Depiction of controlled release strategies in tissue engineering.

becomes denatured may expose sites that are **immunogenic** and lead to the formation of antibodies against the protein that is being therapeutically delivered. The unintended generation of antibodies may not only neutralize the favorable biological interactions between the protein and its target but could also generate a highly undesirable immunological response. As such, one must pay a great deal of attention to the details of the released molecule, to ensure that it is not only released but that it is also released in a native form and with the desired kinetics.

Growth factors are currently the biologics of highest interest in tissue engineering. These molecules are ligands for cell-surface receptors that turn on complex cascades of signal transduction within the cell. Many, but not all, of the growth factors are dimers and bind to receptor tyrosine kinases. Typically, each monomer of a dimerized growth factor binds to the extracellular domain of a receptor, inducing dimerization of the receptor, which leads to phosphorylation of the intracellular domains of the receptor. The resulting phosphorylation of the cytoplasmic region of the receptors leads to altered binding of other intracellular proteins, which then triggers the signal transduction cascade. Refer to [Chapter 4 Cell signaling](#) for more information about cell signaling.

The use of tissue-engineered constructs for immunological purposes is a burgeoning area of research in the biomedical field. The proteins that play a crucial role for the signaling of immune cells are **cytokines** and **chemokines**. The signaling occurs in much the same way as growth factors, but cytokines, in general, stimulate immune cell activation, differentiation, and proliferation, while chemokines direct cell migration. The methods used to control the delivery of growth factors can also be applied for cytokines and chemokines.

Examples of growth factors that are important in tissue engineering are BMPs, TGF- β s, the PDGFs, the fibroblast growth factors (FGFs), the neurotrophins, VEGFs, and the insulin-like growth factors (IGFs)—a nonexhaustive list. The plural is used in the list for the names because the names represent families of proteins; for example, the angiogenic growth factors of the VEGFs consist of VEGF-A, VEGF-B, VEGF-C, and VEGF-D, and moreover, several **isoforms** of these growth factors exist, such as VEGF-A₁₁₀, VEGF-A₁₂₁, VEGF-A₁₆₅, and VEGF-A₁₈₉. The different family members are related by partial

sequence homology and sometimes by overlap in receptor specificity but may have vastly different function. For example, VEGF-A primarily induces angiogenesis (i.e., the formation of new blood vessels from existing ones), whereas VEGF-C primarily induces lymphangiogenesis (i.e., the formation of new lymphatic vessels). The different isoforms are typically different **splice variants** of the same parent sequence and may also have a different signaling capacity. For example, VEGF-A₁₆₅ binds to both VEGF receptors 1 and 2 (among other receptors) as well as to several extracellular matrix (ECM) proteins and cell-surface heparan sulfate, whereas VEGF-A₁₂₁ binds to VEGF receptors 1 and 2, but not to ECM proteins and cell-surface heparan sulfate, which leads to very different biological activities between the two isoforms.

Some of the growth factors mentioned earlier have already been turned into commercial tissue engineering products, for example, PDGF-BB into a product for healing chronic wounds in the skin (Regranex, from Johnson & Johnson) and bone morphogenetic protein-2 (BMP-2) for healing surgical and traumatic defects in bone (e.g., INFUSE, from Medtronic Sofamor Danek). In both cases, prolonged delivery of the growth factor is key to efficacy. In the case of Regranex, frequent readministration is needed for long-term delivery; in the case of INFUSE, this is partially achieved by affinity between the growth factor and a biomaterial matrix, as will be discussed in [Section 12.5](#). However, the clinical translation of these growth factors has been disappointing,^{1,2} and it additionally faces issues related to safety and cost effectiveness.^{3–5} For example, PDGF-BB has not been widely adopted as a main therapy, and the United States Food and Drug Administration (USFDA) recently issued a warning regarding its use related to cancer risk.⁶ These problems derive most likely from the fact that the growth factors are used at vastly **supraphysiological** levels. Therefore, design of controlled release strategies for dose reduction and localization is an ongoing challenge.

Adhesion factors are also important candidates for drug delivery in tissue engineering. These molecules are normally components of the ECM and serve a role of mechanical signaling between the cell's cytoskeleton and the ECM (see [Chapter 5 Extracellular matrix as a bioscaffold for tissue engineering](#)). The **extracellular domains of transmembrane receptors**, typically integrins, bind to the adhesion factors, and the intracellular domains interact, via several other intermediary proteins, with the cytoskeleton. Since mechanical signaling is the ultimate function of receptor ligation, clearly the adhesion factor must be immobilized in the extracellular milieu. As such, these factors function as agonists (i.e., inducing a function in its desired form) in the immobilized state, and as competitive antagonists in their diffusible state. Thus, in the case of these molecules, controlled release means controlled immobilized presentation.

Proteins that contain an array of adhesion factors include fibronectin, vitronectin, fibrinogen, osteopontin, the laminins, and thrombospondin—also a very nonexhaustive list. In addition to the whole proteins, specific integrin-binding domains have been identified within these proteins, and these smaller domains can be recombinantly produced and employed within a controlled release matrix or scaffold. This has been extensively done with fibronectin, which contains multiple independently folding protein domains, many of which bind to integrins.^{7–9} Moreover, still smaller receptor-binding peptides sequences within those protein domains have been identified and successfully used as adhesion factors. Among these, the most widely studied in tissue engineering is the tripeptide sequence Arg-Gly-Asp, or **RGD** in the one-letter code, from fibronectin and several other proteins,

which bind to the integrin family of adhesion receptors.¹⁰ Although the very small RGD sequence binds to a few integrins without a great deal of specificity between integrins, extending the sequence with flanking amino acids or conformationally constraining it by cyclizing the sequence can both increase its affinity for the receptor and recreate some of the specificity between different integrin receptors that exist in the proteins themselves. Several other adhesion factors are under commercial development in tissue engineering now, including members of the laminin family, vitronectin, and decellularized endogenous matrix.

12.2.2 Modes of controlled release

Drugs may be incorporated within biomaterials in a homogeneous or a heterogeneous manner, as shown in Fig. 12.3A. When incorporated in a homogeneous manner, an implicit requirement is that the drug be somehow soluble in the matrix material, or at least soluble in its precursors. In this way,

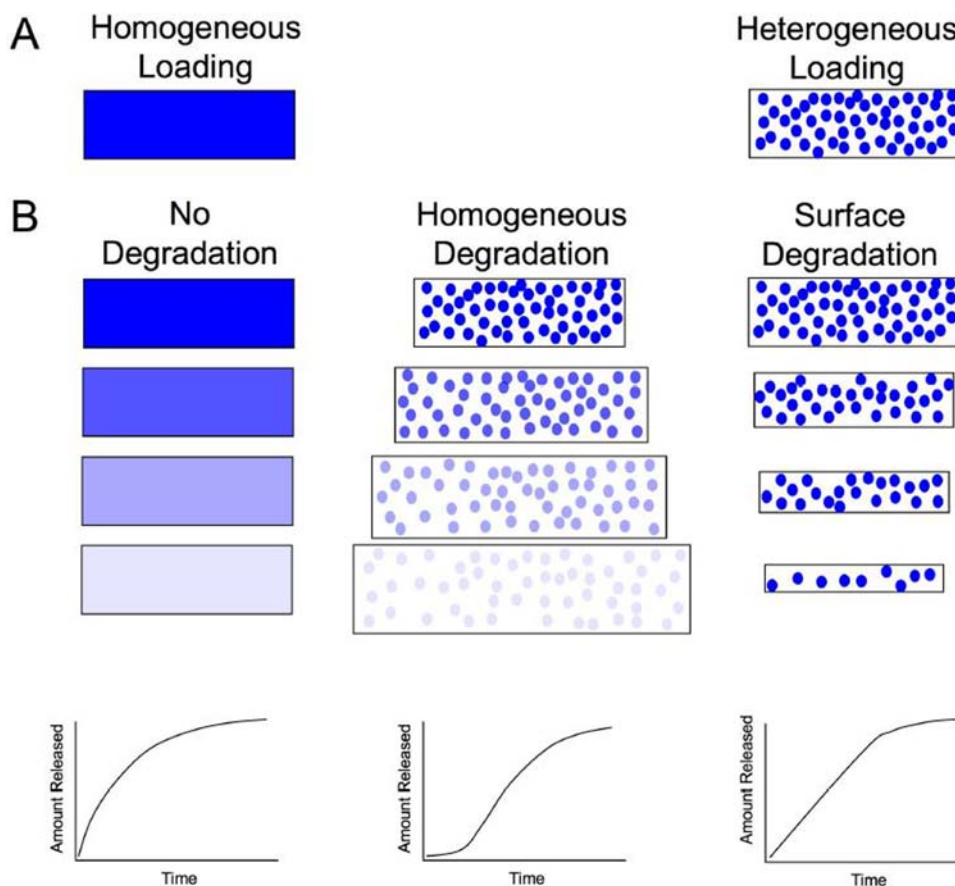


FIGURE 12.3

Time release profiles of homogeneous and heterogeneous drug loading in matrices.

the drug can be homogeneously dissolved in the biomaterial, and, although a solid, the matrix acts as a solvent for the drug; the entire complex of drug and matrix is referred to as a **solid solution** of the drug within the matrix biomaterial. Given that the drugs of interest in tissue engineering are typically water soluble and macromolecular, this situation usually occurs only when the biomaterial is very hydrophilic. Such biomaterials then absorb large amounts of water and are referred to as **hydrogels**. Hydrogels may either be microporous, for example, composed of hydrophilic nanoscopic fibrils or cellular structures, such as a fibrin hydrogel, or they may be nanoporous, composed of a homogeneous network of water-soluble synthetic polymer chains cross-linked into a solid by covalent or physicochemical bonds.

When hydrophobic polymers are used, that is, those not forming hydrogels, the drugs that are of most interest in tissue engineering are not soluble within them and they form a heterogeneous structure. Domains of the drug are dispersed throughout the biomaterial scaffold heterogeneously as nanodomains or microdomains. This situation would exist with polymers such as the degradable polyesters poly(lactic acid), poly(glycolic acid), and poly(ϵ -caprolactone) and their copolymers. If the protein drug were incorporated within the polymer scaffold as a dry powder, such as produced by spray drying or by lyophilization, then pools of dry or highly concentrated protein would exist dispersed throughout the scaffold. It is also possible to incorporate the protein drug as an aqueous solution dispersed throughout the polymer scaffold, using an emulsion process; this results in a polymer scaffold with pools of dissolved protein drug dispersed heterogeneously throughout as shown in [Fig. 12.3A](#).

Just like there is more than one mode of drug incorporation (homogeneous, heterogeneous), there is also more than one mode of drug release, depending upon the mode of drug interaction with the biomaterial and the mode of material degradation, as shown in [Fig. 12.3B](#). A first mode of release may be found in the case of hydrogel matrices that have mesh sizes that are sufficiently large as to not present a substantial resistance to drug diffusion, for example, mesh sizes larger than approximately 100 nm. In the case of highly porous hydrogels, the drug is not entrapped within the matrix, and drug release thus proceeds independently of biomaterial degradation; the biomaterial may degrade quickly, or slowly, or not at all, but drug release proceeds more or less independently of these considerations. Such a situation is referred to as **diffusion-controlled release**. In this case, the **diffusion coefficient** of the drug in the hydrogel matrix is constant over time since the hydrogel structure does not change during the process of release. Since the concentration of drug remaining in the hydrogel matrix decreases over time, due to the release process, the rate of release decreases over time, and a constant rate of release is unachievable. When relatively short-term release characteristics are desired, this approach may be appropriate. The biomaterial plays its main role as a vehicle for local, if transient, administration of the drug. The biomaterial may also play a second role, as a matrix for cell transplantation or cell invasion, independent of its role in drug release.

A second mode of release may be found in the case of hydrogel matrices with smaller mesh sizes. The polymer network that composes the gel may efficiently entrap the protein drug and hinder its diffusion from the material. In this case, the drug cannot escape from the matrix until the matrix begins to degrade. As **hydrolysis** occurs in the material, the architecture of the polymer network is cleaved, and results in an increase in the mesh size and an increase in the overall swelling of the material. As this occurs, the drug becomes less entrapped and may begin to diffuse from the matrix. Given that hydrolysis occurs throughout the material and that drug release then ensues from the entire material, this

situation is referred to as **homogeneous degradation**. Such situations would exist in a uniform hydrolytically degradable hydrogel, such as a hydrolytically degradable polyethylene glycol (PEG).¹¹ Whenever water is in the hydrogel structure, hydrolysis occurs. Since the hydrogel is highly swollen with water, water is equally present throughout the hydrogel matrix, and as such degradation proceeds equally fast throughout the matrix. This leads to porosity of the hydrogel network that increases over time, and then diffusive release of drug that correspondingly increases over time. In this case, the diffusion coefficient of the drug in the hydrogel matrix starts out at a very low value, too low to demonstrate substantial release. As the gel degrades, the diffusion coefficient increases over time, and this leads to increasing release rates. However, as release occurs, the concentration of the drug remaining in the hydrogel matrix decreases over time, somewhat offsetting this increasing release rate. When these two effects are balanced, release characteristics can be obtained that are nearly *zero order*; that is, they are nearly constant over time. The extent to which this is true depends upon the extent to which the two opposing effects (diffusion coefficient increasing over time and concentration in the matrix decreasing over time) are appropriately counterbalanced. When prolonged release is needed, for example, for slow morphogenetic processes, this mechanism of controlled release can be very appropriate.

A third mode of release may be found with the use of more hydrophobic materials. In the case of such hydrophobic polymers used as biomaterial scaffolds, water will naturally have much better access to the surface of the scaffold than the internal domains of the scaffold; this leads to polymer hydrolysis in a heterogeneous manner, first on the surface, and then deeper in as the surface erodes and exposes new polymer to the surface, much like a bar of hand soap dissolving. Such degradation is referred to as **surface degradation**. With surface degradation, the drug is likewise released from the biomaterial scaffold layer by layer, and the rate of drug release depends strongly on the rate of polymer degradation and on the morphology of the implant. For example, the surface area of a polymer film does not change very much as the film degrades, and thus, the rate of release in such morphologies is close to constant over time, which is referred to as **zero-order release**. In cases of release by surface degradation, it is easier to achieve zero-order release than with other release mechanisms.

Finally, a fourth mode of controlled drug release may be found when the bioactive molecule is not desired to be released at all, for example, of **biomolecule immobilization**. The principal example of when this is desired is with adhesion proteins and peptides, which are active in their bound state, rather than free. Here, both surface and bulk immobilization must be considered. If an adhesion molecule is being immobilized on the surface of a surface-degrading scaffold, to support cell attachment or to trigger cell differentiation, it must clearly remain on the surface for the desired duration. If the surface layer is removed from the scaffold too rapidly, then the biomolecule will not be present sufficiently long to carry out the desired signaling function. In this case, it would have to be immobilized throughout the bulk of the material, so that when one polymer surface layer is degraded, new adhesion molecules are exposed. If the scaffold degrades slowly, compared with the time scale of cell differentiation and tissue formation within the scaffold, then these considerations are less important. Likewise, if the scaffold degrades by bulk degradation, then the rate of removal of the adhesion molecule from the polymer surface will likewise be slow, even if it is not immobilized throughout the bulk. Thus, the necessity of immobilization throughout the bulk, or the satisfactory nature of surface immobilization, depends on the details of the application and the material.

Having now introduced the topic to consider what types of drugs are interesting to release (mostly proteins and peptides), and the different modes of controlled release that may be encountered (diffusive release, release with bulk or surface degradation, no release at all), we can now turn our attention to specific examples, as indicated in Table 12.1.

12.3 Physical mixtures of bioactive factors within matrices

In some cases, the proteins that form biomaterial matrices and scaffolds may serve directly as a carrier for controlled delivery of a bioactive molecule. Several possibilities exist by which the scaffolds may prolong release of diffusible protein drugs such as growth factors. The scaffold or matrix may merely serve to localize the placement of the drug as a diffusion source, as described earlier regarding diffusion-controlled release. Alternatively, even a relatively small affinity between the biomaterial matrix and the factor may result in a useful prolongation of release, as has been observed with gelatin and collagen matrices.¹² Finally, for drugs that display a relatively poor solubility, the matrix or scaffold can contain a continuous source of slowly dissolving factor and provide sustained release for an effective duration.

Type-I collagen makes up a large fraction of connective tissues, such as skin and tendon, and bovine skin from **closed and controlled herds** makes up a useful supply of type-I collagen for use as biomaterial matrices in tissue engineering.^{13–15} The material may be used in granular form, as is used in the commercial product OP-1 Implant in the United States and in Europe (Stryker Biotechnology), which consists of osteogenic protein-1 (OP-1, also known as bone morphogenetic protein-7 or BMP-7) dispersed in granules of type-I collagen. This product has been approved for clinical use in **tibial non-unions** after failed **autograft**. Bovine type-I collagen may also be used in the form of a **macroporous** sponge as a drug delivery and cell ingrowth matrix. Such sponges may be placed in bony defects, as well as within medical devices such as **spinal fusion cages**, which are hollow structural devices used to maintain the spacing between the vertebral bodies when the intervertebral disc is removed and bony fusion between the two vertebrae is sought. BMP-2 has been developed as a product for such bone growth applications, in the product INFUSE in the United States and InductOs in Europe (Medtronic Sofamor Danek). BMP-2 in such collagen sponges has been shown to remain at the implantation site in vivo for at least 2 weeks, using radiolabeled protein.¹⁶

BMPs in collagen matrices have gone forward to clinical testing in humans, after substantial testing in preclinical animal models.^{16–18} For example, BMP-2 in bovine type-I collagen sponges was evaluated in two clinical studies^{19,20} applied within cylindrical interbody spinal fusion cages. This compared the cage plus the BMP-2 in a collagen sponge with the standard of care at the time, which was the spinal fusion cage with bone autograft taken from the iliac crest. Bone formation in and around the cage is illustrated in Fig. 12.4. The BMP-2-treated group (Fig. 12.4A) performed as well in terms of fusion between the two vertebrae as the group treated with standard of care (Fig. 12.4B). The controlled release of BMP-2 from collagen sponges demonstrated a substantial advantage by negating the need to harvest bone from the iliac crest, a procedure that is associated with significant morbidity.

In the earlier examples, we see that bovine type-I collagen can be used successfully as a carrier of growth factors in tissue engineering, such as BMPs in bone tissue repair. However, due to the poor affinity

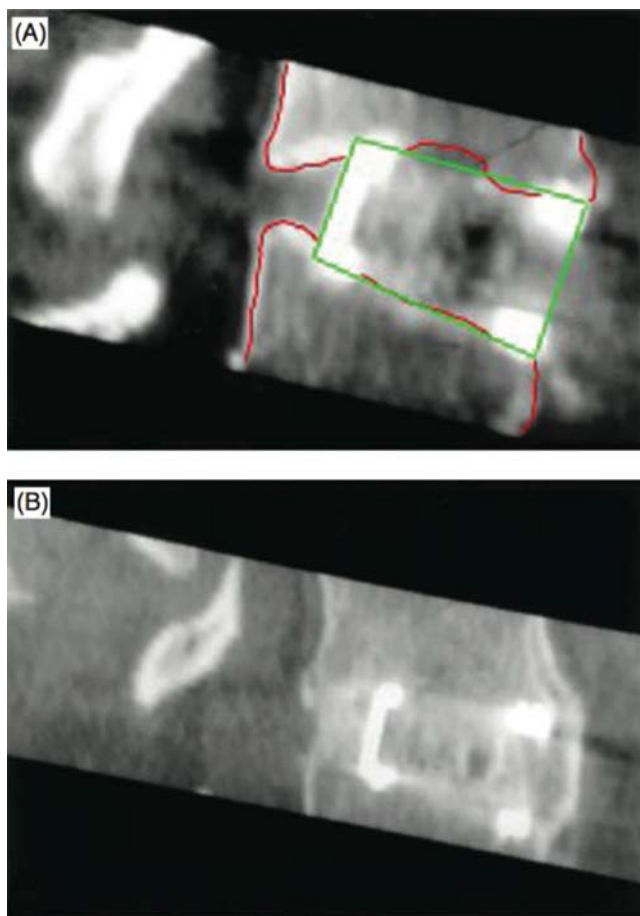


FIGURE 12.4

Radiographic images of vertebrae treated with a spinal fusion cage filled with Bmp-2. From Burkus JK, Dorchak JD, Sanders DL.

Radiographic assessment of interbody fusion using recombinant human bone morphogenetic protein type 2. Spine 2003;28:372–377.

between BMPs and type-I collagen, the factors are not retained in the matrix for very long, a fact that requires the use of supraphysiological doses of the growth factors in the successful therapies. The use of very high doses has caused safety issues, for example, the safety of BMP-2 in INFUSE for bone repair has been called into question,³ and the use of BMP-2 for posterolateral spinal fusion showed clinical incidences of cancer that were of substantial concern to the USDA (2010). In addition to the lack of highly prolonged delivery, there may be immunological consequences of the use of bovine proteins. For example, in the clinical evaluation of BMP-7 in bovine type-I collagen granules, 5% of the patients developed antibodies for the bovine collagen, and 10% of the patient's developed antibodies against the recombinant human BMP-7.²¹ One could hypothesize that the bovine collagen acted as an adjuvant

to enhance immune reactions against the recombinant human protein, and these worries create a driving force for the development of either human-derived or synthetic matrices that would function as well.

Human-derived matrix proteins are being developed as an alternative to bovine-derived proteins. Although human collagen is available for clinical use (e.g., purified from placentas), its expense is such that it is not widely used for implants that require high masses of protein. Rather, human fibrin is widely available and broadly utilized, purified from human blood plasma donations.²²

Prolonged release of BMP-2 has been achieved in gels with open networks, such as fibrin and collagen, which do not provide a substantial spatial barrier for protein diffusion. Recombinant BMP-2 when produced in mammalian cells is **glycosylated**, whereas BMP-2 produced in engineered bacterial cells is not glycosylated. The glycosylation of BMP-2 (and other proteins) increases its solubility as shown by the diamond data points in Fig. 12.5. The nonglycosylated, bacterially produced BMP-2 is much less soluble than mammalian cell-produced BMP-2 as shown by the square data points in Fig. 12.5. When the nonglycosylated protein was complexed with heparin (circles in Fig. 12.5), its release was accelerated and became as fast as the glycosylated protein. This has been utilized to produce BMP-2 that is retained in fibrin matrices for prolonged periods relative to mammalian cell-produced BMP-2, which demonstrated in clinical studies in dogs (veterinary patients) healing in bone fusions that was statistically equivalent to that obtained with bone autografts.²³

A typical example of healing in these animals is illustrated in Fig. 12.6, showing nonglycosylated BMP-2 used in fibrin surgical matrices to fuse the carpal joint complex in canine patients in **pancarpal**

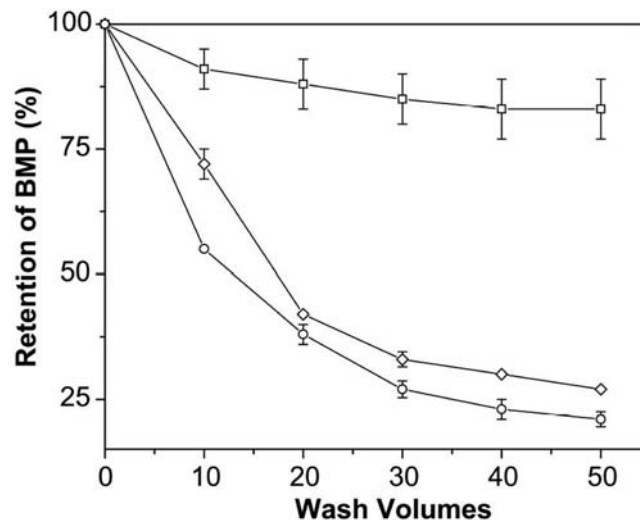


FIGURE 12.5

Retention profile of glycosylated and nonglycosylated BMP-2 from fibrin with and without heparin. From Schmoekel H, Schense JC, Weber FE *et al.* Bone healing in the rat and dog with nonglycosylated BMP-2 demonstrating low solubility in fibrin matrices. *J Orthop Res* 2004;22: 376–381.

**FIGURE 12.6**

Nonglycosylated BMP-2 used in fibrin surgical matrices to fuse the carpal joint complex in canine patients in pancarpal arthrodesis procedures. From Schmoekel H, Schense JC, Weber FE et al. Bone healing in the rat and dog with nonglycosylated BMP-2 demonstrating low solubility in fibrin matrices. *J Orthop Res* 2004;22:376–381.

arthrodesis procedures. Normally, the joint is stabilized with a surgical plate (shown on the radiographs), and autologous bone is implanted within the joint space to induce joint fusion. Here, fibrin with nonglycosylated BMP-2 was injected in the joint space, rather than bone autograft. Panel (A) is taken immediately after the procedure; no bone is visible within the three joints of the complex (arrowheads) (Fig. 12.6A). In panel (B), after healing, the joints were completely fused, as demonstrated by radio-opacity within the joint space (arrowheads) (Fig. 12.6B). In a study comparing nonglycosylated BMP-2 in fibrin, arthrodesis was as effective as in a control cohort treated with both autografts. This principle of controlled release would presumably also work in other highly porous biological matrices, such as type-I collagen matrices.

As an alternative to native protein matrices being used as admixtures with protein drugs such as growth factors, denatured protein matrices can also be used. The main example of this is bovine-derived gelatin. Gelatin is produced by **denaturation** of type-I collagen, and an unfolded protein ensemble that self-assembles into a gel by hydrophobic interactions is obtained. These gels, nearly identical to

that of edible gelatin, are not very stable at physiological temperatures, and one can achieve more stable gels by chemical cross-linking with bifunctional agents, such as glutaraldehyde, which reacts with amine functionalities in the denatured protein chains coming mostly from lysine residues. Cross-linked gelatin matrices have been widely used in animal studies with proteins such as BMP-2, both with and without inorganic particles of tricalcium phosphate to assist in stability during bone formation.^{24,25} In this case, it is likely that the controlled release of the growth factor within the gelatin matrix is due to many, nonspecific, low-affinity, electrostatic attraction with the gelatin matrix. This has been more clearly demonstrated in a study with two hepatocyte growth factor (HGF) isoforms interacting with gelatin. In this study, native HGF was demonstrated to be released very slowly from cross-linked gelatin matrices, almost as slowly as material degradation. By contrast, when a highly charged region of the protein was deleted to make a less charged variant protein, that variant was released by simple diffusion from the matrix, much faster than the native isoform.²⁶ This demonstrates an additional principle for controlled release in highly porous biopolymer matrices and the use of numerous, but low-affinity electrostatic interactions between the protein drug and the matrix to obtain sustained release.

In addition to low-affinity interactions, some biopolymeric matrices have the potential to exhibit high-affinity biophysical interactions for some protein drugs, especially some growth factors. This principle can be illustrated with fibrin. Fibrin forms in the body at sites of injury, and accordingly some growth factors have evolved to be immobilized in such fibrin clots.²⁷ Cells such as blood platelets are naturally present within fibrin clots, and these cells release large quantities of stored growth factors to induce healing. Several growth factors such as placenta growth factor-2 (PlGF-2) and fibroblast growth factor-2 (FGF-2), which contain very high-affinity binding sites for fibrinogen and fibrin, with a dissociation constant <10 nM.^{28–30} For example, this affinity immobilizes the FGF-2 within fibrin matrices, protects it from proteolytic degradation,³¹ and may potentiate its activity in biological processes such as angiogenesis.³² Fibrin is not the only biological matrix molecule with activity to bind certain growth factors: several other growth factors also bind fibronectin,³³ vitronectin,³⁴ tenascin-C,³⁵ osteopontin,³⁶ type-I collagen,³⁷ and type-II and type-IV collagen.^{38,39}

In this section, we have seen that relatively simple approaches to controlled release of protein growth factors as mixtures in biopolymer matrices can be very useful. Examples of such biopolymers are type-I collagen granules and sponges, cross-linked gelatin matrices, and fibrin. The scaffold or matrix may merely localize the drug, or it may serve to delay release of the drug by nonspecific electrostatic affinity. In the case of matrix-growth factor pairs, such as fibrin and PlGF-2, naturally evolved high-affinity interactions may result in prolonged retention of the drug within the matrix. Admixtures of growth factors in biopolymer matrices have already resulted in commercial products in tissue engineering, such as the BMPs used in bone repair.

12.4 Bioactive factors entrapped within gel matrices

It is possible to make hydrogel matrices that have characteristics and porosities such that protein permeation is directly inhibited by entrapment. This may be done in two ways: either the network structure of the hydrogel matrix may be sufficiently tight as to resist protein diffusion, or the polymer chains in the network may drive the protein out of solution. Both concepts are described in this section.

Homogeneous hydrogels have a structure not unlike a three-dimensional (3D) fishing net, with polymer chains connecting cross-links within the network, which may be covalent or physical.^{40–42} The molecular weight of the chains that connect the cross-links and the basic structure of the polymer chain determine the distance between the cross-links and thus the average **pore size** within the material.⁴³ These pores should not be thought of as static pores with rigid walls, rather they are formed dynamically and represent a time average, like the porosity created by the strings of a fishing net under water, sometimes looser and sometimes tighter as the polymer chains self-diffuse. When the average distance between cross-links within the hydrogel network approaches the same order of magnitude as the diameter of the protein drug, diffusion of the drug becomes significantly restricted, and the drug becomes entrapped within the hydrogel. This physical restriction can be useful in controlled release in tissue engineering.

Proteins can also be entrapped within small hydrogel particles, and these particles can be distributed throughout a matrix or scaffold to provide a sustained source of a diffusible bioactive factor such as a growth factor. One such hydrogel structure is obtained when water-soluble polymer chains are attached to a polymerizable species at their termini, and then, this macromonomer is further polymerized. An example of this is with PEG, a polymer that is highly soluble in water. This chain can be reacted with ester monomers, to make a still water-soluble ABA **block copolymer**, where the A block is an oligomer of, for example, lactic acid, and the B block is the central PEG chain, as illustrated in Fig. 12.7A.

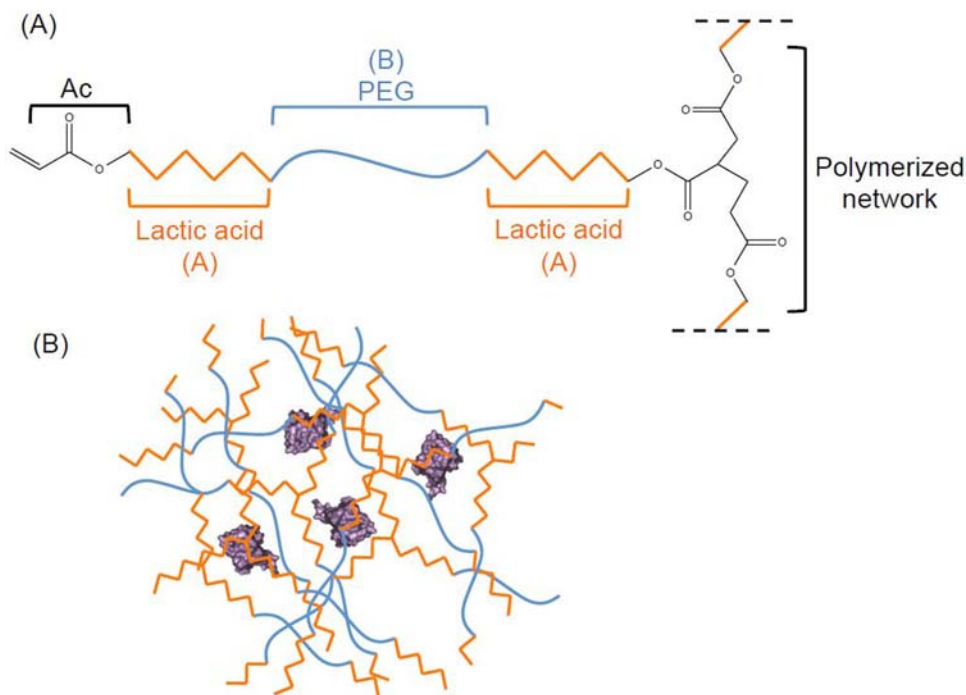


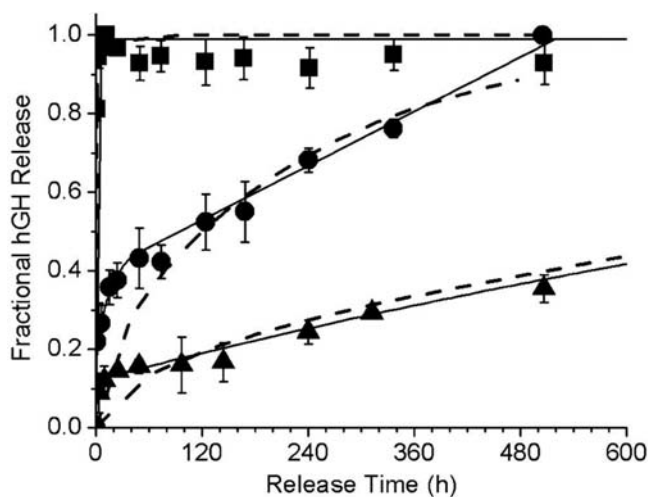
FIGURE 12.7

Formation of hydrolytically sensitive PEG hydrogel networks.

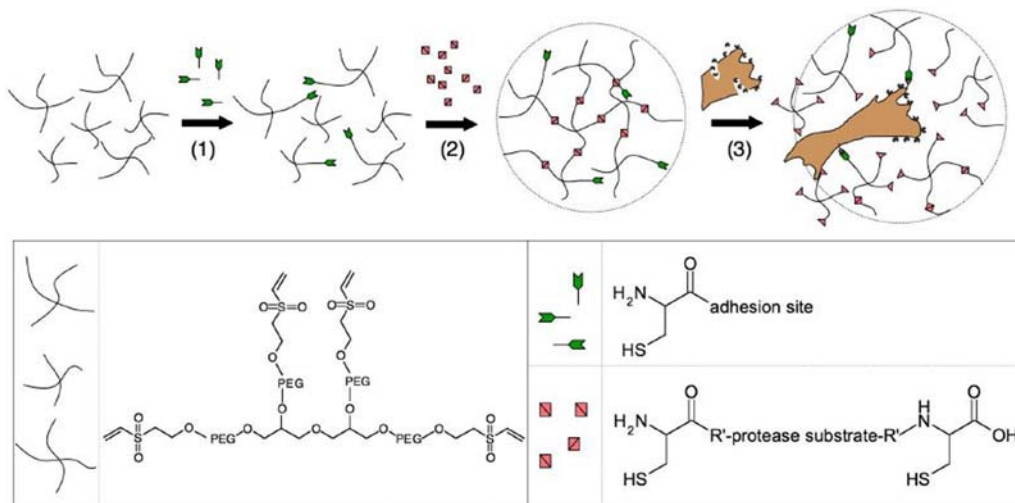
This polymer is still water soluble, and as such, it is not useful in sustained release (unless the A blocks are sufficiently large to drive the entire polymer out of solution). If the chain termini are attached to polymerizable acrylate groups (Ac), to form Ac-oligo(lactic acid)-poly(ethylene glycol)-oligo(lactic acid)-Ac, then the resulting macromonomer can be polymerized to form a cross-linked hydrogel (the cross-links, or knots in the fishing net, are chains of Ac groups attached to each other) connected by PEG chains (the strings in the fishing net), with a hydrolytically sensitive link between the cross-links (the oligo(lactic acid) blocks). Polymerization requires a cross-linker that contains at least three reactive groups to achieve network formation. With only two reactive groups, polymerization would result in only a long linear chain instead of a cross-linked network. The Ac groups can each react with two other Ac groups during photopolymerization, resulting in four reactive groups per ABA block and a highly cross-linkable polymer. Such a network is shown in Fig. 12.7B; when the distance between cross-links is a similar length scale as the diameter of the protein, the protein drug may be entrapped. As the strings in the network degrade, this distance between cross-links increases, and the protein may escape. The polymerization can be carried out in water under sufficiently mild conditions to not degrade proteins incorporated in the hydrogels. By tuning the molecular weight of the PEG blocks, and thus the length between the cross-links within the matrix, the release rate of the entrapped proteins can be modulated.⁴⁴

The incorporation of proteins within a 3D network can be challenging. In the earlier example, the gel was polymerized with the protein dissolved in the macromonomer solution. Other chemistries have improved upon this method; for example, PEG has been further derivatized to form a chemically reactive, branched polymer. Four-arm or an eight-arm PEG starburst has been functionalized with reactive groups on the ends and then cross-linked by the addition of a corresponding chemically reactive group forming a tailored, close-knit hydrogel.^{45–48} Here, the knots in the network are the central portions of the starburst polymers, and the strings between the knots consist of one PEG chain, the cross-linking molecule, and the second PEG chain. Fig. 12.8 shows the release of human growth hormone from different cross-linked networks of PEG hydrogels. The polyethylene chains are reacted with acrylate groups, and these are cross-linked to small molecular weight dithiols such as dithiothreitol or a low-molecular weight PEG diacrylate. The squares show an eight-arm 10 kDa PEG octa-acrylate forming a very loose network, causing the incorporated protein to be released quickly by simple diffusion. However, when the meshwork is formed with shorter PEG chains, eight-arm, 2 kDa PEG octa-acrylate, degradation-controlled release can be obtained (triangles). A 50%:50% blend of the two (circles) shows the release from an intermediate network.

In the earlier examples, sustained release was obtained when the network size was of the same order of magnitude as the protein diameter. If this is the case, of course, the network porosity is very small relative to the size of a cell, and cells are unable to penetrate the hydrogel network. Therefore, these sorts of delivery vehicles would commonly be presented as microparticles dispersed through another scaffold or matrix. An alternative is to endow the **nanoporous** network with peptide cross-links that can be enzymatically degraded by migrating cells, thereby releasing the entrapped morphogens.^{49–52} This is illustrated in Fig. 12.9. As depicted, biomimetic gel matrices can be constructed by cross-linking of reactive branched PEGs, e.g., terminated with vinylsulfone moieties, and di-thiol peptides (red squares), with a cysteine residue on each end of the peptide. Because the PEG chain contains more than two reactive groups, and the peptide contains two, a cross-linked network forms in step 2 of the reaction. In step

**FIGURE 12.8**

Controlled release via the network architecture of cross-linked PEG hydrogels. From van de Wetering P, Metters AT, Schoenmakers RG, Hubbell JA. Poly(ethylene glycol) hydrogels formed by conjugate addition with controllable swelling, degradation, and release of pharmaceutically active proteins. *J Contr Release* 2005;102:619–627.

**FIGURE 12.9**

Design of biomimetic gel matrices degradable by migrating cells. From Lutolf MP, Raeber GP, Zisch AH, Tirelli N, Hubbell JA. Cell-responsive synthetic hydrogels. *Adv Mater* 2003;15:888–892.

1, an adhesion peptide, such as the RGD peptide, can be incorporated into the network as a peptide that contains a single cysteine residue. If the cross-linking peptide is designed as a substrate for cell-expressed enzymes, such as matrix metalloproteinases (see [Chapter 5 Extracellular matrix as a bioscaffold for tissue engineering](#)), then cells can locally degrade the matrix and migrate through it, as in step 3 of the reaction. Given that the enzymes are localized near the surfaces of the cells, matrix degradation occurs only at the surface of the cells as well, degrading the matrix as the cells migrate through it.

These hydrogel matrices offer a great deal of design flexibility: if peptides are designed to be fast-degrading substrates, then cell migration through materials can be very fast, and if slower-degrading substrates are used, then cell migration and material degradation, and thus protein release, are slower. In one example, shown in [Fig. 12.10](#), BMP-2 was incorporated into such matrices and implanted in bone defects in rats, bone repair was induced as shown by the blue-green regions, and cell infiltration in red. All gels contained RGD peptides as an adhesion moiety. The gels that contained matrix metalloproteinase sensitive cross-links and BMP-2 demonstrated matrix infiltration and good bone

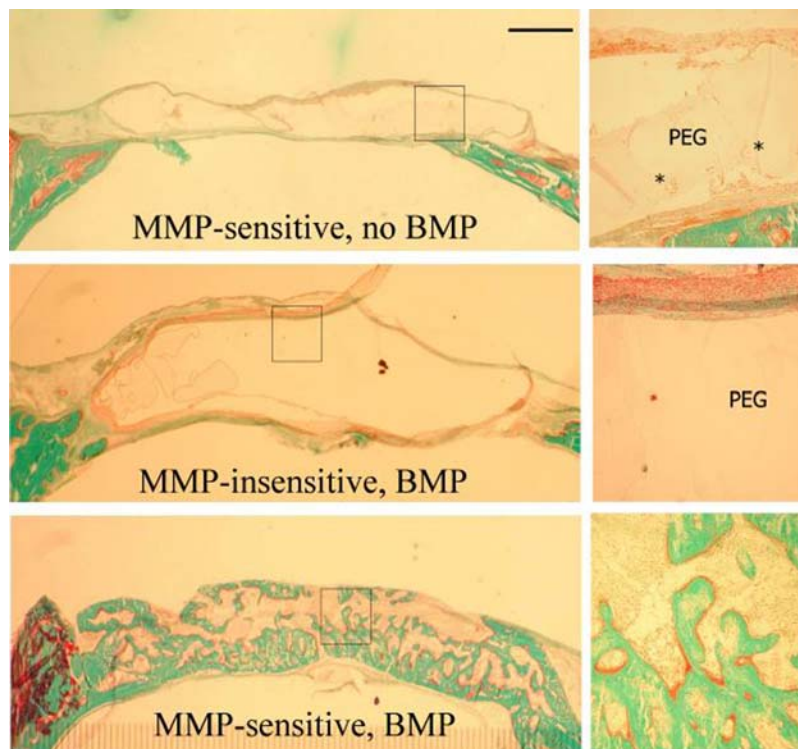


FIGURE 12.10

Bone healing of critical-size rat calvaria defects with MMP-sensitive and adhesive PEG gels containing BMP. From Lutolf MR, Weber FE, Schmoekel HG et al. *Repair of bone defects using synthetic mimetics of collagenous extracellular matrices*. Nat Biotechnol 2003;21: 513–518.

formation as shown in the lower panel. Such a release concept can be very advantageous for morphogenesis into a matrix, in that the rates of release of the morphogen are automatically linked to the rate of cell infiltration into the matrix.

In this section, we have seen that proteins, which are essentially hydrophilic in nature, can be entrapped within hydrophilic networks. The network structure, if sufficiently small, can resist the diffusion of the protein. The controlled release of proteins from these networks can be achieved through severely restricted diffusion or through degradation of the network. The network degradation can be hydrolytic, or it can be proteolytic.

12.5 Bioactive factors entrapped within hydrophobic scaffolds or microparticles

Degradable hydrophobic polymers such as poly(lactic acid), poly(glycolic acid), poly(ϵ -caprolactone), and their copolymers have been widely explored for the controlled release of proteins, for applications that include tissue engineering. These polymers can display distinct advantages for many applications; these advantages include the long duration over which controlled release may be sustained and the ability to integrate drug delivery directly into a structural scaffold built from the same polymer. Since the polymer is hydrophobic and the drug is usually hydrophilic, the drug is distributed heterogeneously throughout the polymer, and it escapes as the polymer degrades. In the following paragraphs, we describe several means by which protein drugs have been incorporated into hydrophobic polymers, either as microparticles distributed throughout a scaffold or within a scaffold itself directly.

One of the easiest ways to incorporate a protein drug within a polymeric scaffold is by simple adsorption to the scaffold surface. Although this does not allow great control over the release characteristics of the drug, useful sustained release and efficacy can sometimes be obtained when the therapeutic window of the drug is relatively wide. A good example has been provided in scaffolds intended for bone regeneration.^{53–55} Polylactide-*co*-glycolide (PLGA) microparticles, 600–700 μm in diameter, were compressed within a metal mold, which was then heated to a temperature more than the polymer's **glass transition temperature**. At such temperatures, **molecular mobility** within the polymer is enabled, to allow chains from one microparticle to diffuse into the surface of neighboring microparticles. When the system is cooled, this gives a sintered solid, with porosity remaining from the interstitial space between the microparticles. This creates a very high surface area within the implant per unit volume. This surface can be used to adsorb protein, at surface concentrations up to 100–500 ng/cm^2 . Given that many growth factors are active at amounts from 10 to 100 ng, this can represent a large quantity of the growth factor. One favorable feature of such porous solids is that they can be very strong, and thus can be used as structural implants in the repair of load-bearing tissues, such as bone. The incorporation of BMP-7 within porous sintered solids demonstrated very effective bone healing in rabbits. When cells from the bone marrow were also seeded within the scaffolds, even more effective bone healing was observed. This example demonstrates that growth factor can be incorporated by simple means such as adsorption (because very little of the growth factor is in principle required), that the drug delivery vehicle can be the same as the scaffold, and that such approaches can be useful for cells invading a matrix as well as in differentiation of cells that are transplanted into a matrix.

More sophisticated approaches for drug incorporation can also be undertaken, with correspondingly better control of the release characteristics of the construct. For example, the protein drug can be formulated as a dry powder in a finely distributed state, such as by **lyophilization** into a fine cake and then **micronizing** that cake into small particles in a ball mill. Alternatively, the protein can be spray dried directly into fine particles. Processes have been described for the incorporation of such powders directly into polymer scaffolds,⁵⁶ see the text box describing the seminal work by Robert Langer on the topic.

In addition, morphogens, such as BMP-7, have been suspended in degradable polyester polymer in an organic solvent, films were cast, and the solvent was evaporated to result in fine particles of the protein drug distributed throughout the polymer films. When these films with BMP-7 were seeded with cells isolated from muscle, the growth factor enhanced their osteogenic differentiation, and these cell-seeded polymer films were useful in repair of bone in rabbit models. This example demonstrates that protein drug can be loaded as a particulate discontinuous phase within the degradable hydrophobic polymers, and that these constructs can be useful in cell differentiation and tissue repair.

Methods such as those described earlier can be used with a variety of polymeric scaffolds in tissue repair, even in rather complicated conditions. For example, angiogenic growth factors such as VEGF-A can be incorporated into polymeric scaffolds formed by a gas foaming method.⁵⁷ When the VEGF-A was incorporated into the surface of the polymer, it was released quickly from the implant. However, if VEGF-A was preencapsulated within degradable hydrophobic polyester microparticles, which were then incorporated into the scaffold, release was substantially delayed.⁵⁸ Such **composite polymer systems**, showing distinct release kinetics for growth factors, are useful since tissue repair and regeneration involve the sequential signaling of multiple growth factors (Fig. 12.11). Proper engineering matrices for control release of biomolecules enable **spatiotemporal delivery** of multiple growth factors. This can be achieved by creating biomaterial systems showing distinct release kinetics for growth factors. The matrix system can be designed to display different affinities or physical barriers for distinct growth factors, or the growth factors are engineered to display different affinities for the matrix producing different release

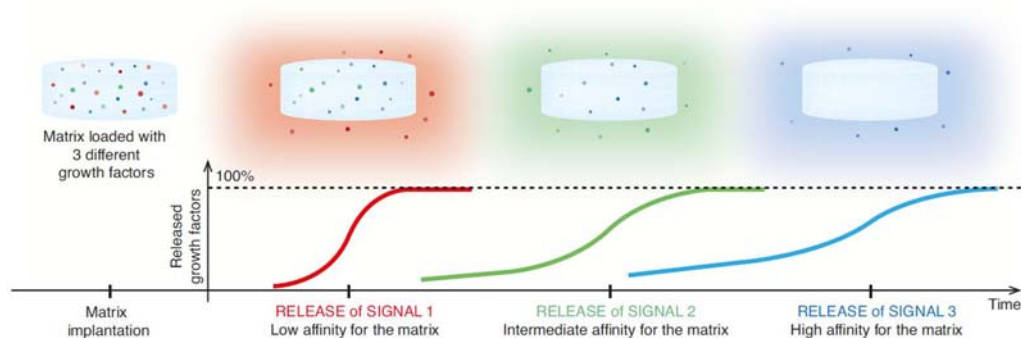


FIGURE 12.11

Variable release profiles for improved tissue regeneration. From Rice JJ, Martino MM, De Laporte L, Tortelli F, Briquez PS, Hubbell JA. *Engineering the regenerative microenvironment with biomaterials*. Adv Healthcare Mater 2013;2:57–71.

profiles. The system controls the temporal release of distinct growth factors resulting in sequential waves of signaling, which can control various stages of the healing process, beginning with the initial inflammatory response and cell recruitment, leading to cell differentiation and tissue morphogenesis, and finally maturation of the tissue.

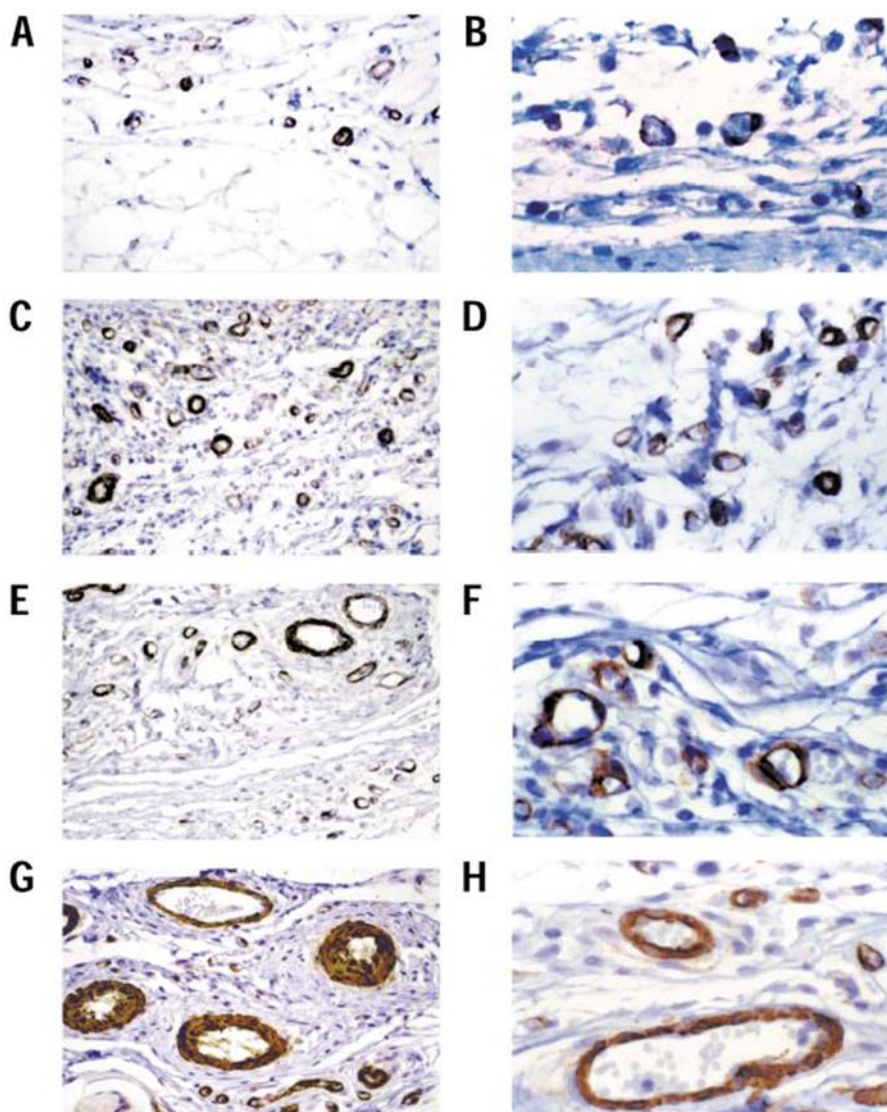


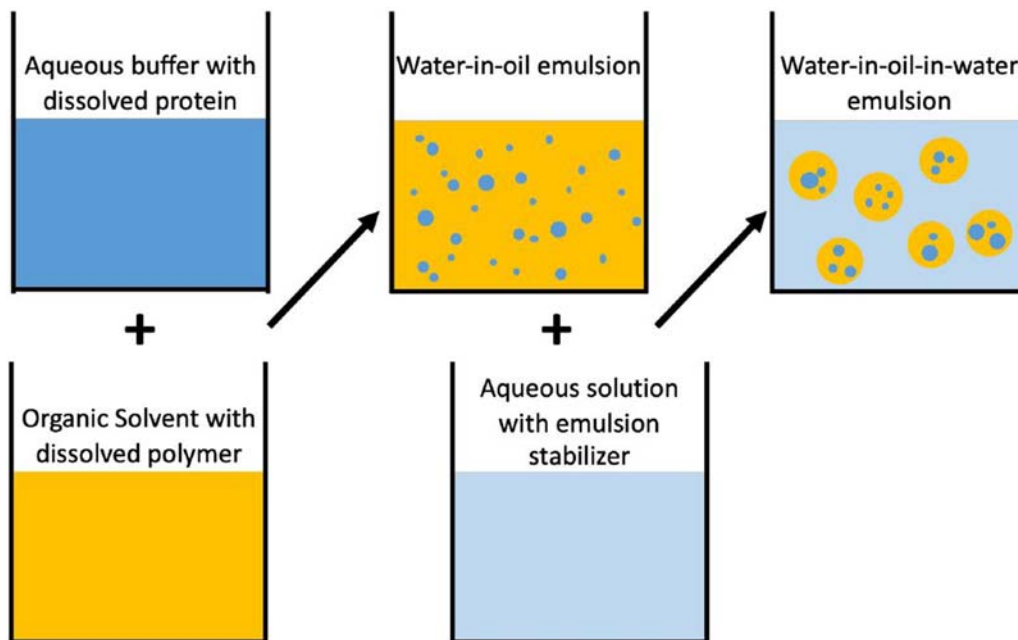
FIGURE 12.12

Enhanced angiogenesis using dual growth factor (VEGF and PDGF) release systems. *From Richardson TP, Peters MC, Ennett AB, Mooney DJ. Polymeric system for dual growth factor delivery. Nat Biotechnol 2001;19:1029–1034.*

This difference in release rates has been strategically used to promote angiogenesis.⁵⁹ The growth factor VEGF-A induces angiogenesis; however, the newly formed blood vessels are often immature, as they are hyperpermeable and unusually branched in architecture. Other growth factors, such as PDGF, are known to induce maturation of blood vessels, by associating the vessels with other cells that stabilize and reduce the permeability of the newly formed vessels. This two-stage release method was used to release VEGF-A quickly, which is incorporated into the surface of the scaffold, and PDGF more slowly, which is preincorporated into microparticles that were then incorporated into the scaffold. When angiogenesis was studied in animal models, substantially more mature angiogenesis was obtained with the dual growth factor release system than with the single growth factor release system releasing VEGF-A.⁵⁹ The difference in the maturity of newly formed vessels with dual release is illustrated in Fig. 12.12. Here, α -smooth muscle actin was stained brown as an indication of vessel stabilization by smooth muscle cells. Figs. 12.12A and B show minimal angiogenesis within scaffolds containing no growth factors. Figs. 12.12C and D show some angiogenesis within scaffolds containing only VEGF. Figs. 12.12E and F show a little more angiogenesis within scaffolds containing only microencapsulated PDGF. And finally, Figs. 12.12G and H show excellent vascularization when scaffolds release both VEGF and PDGF. These results demonstrate a vascular response (shown by the circular cross section of blood vessels) that was higher than strategies using one growth factor. More vessels (stained positive for both endothelial cell and smooth muscle cell markers -brown circles-) are visible.

It is instructive to think about the details of how protein drugs can be incorporated into polymer microparticles. The most common method is referred to as the water-in-oil-in-water emulsion method.⁶⁰ The final structure consists of a polymer microparticle with dispersed domains of protein-containing water droplets within it. It is also possible in some circumstances to further dry the watery domains so that they consist of merely dry protein dispersed throughout the polymer microparticle.

The construct is made as follows (Fig. 12.13): in one preparation, the protein is dissolved in a buffer (dark blue), which is usually optimized for long-term stability of the protein to be encapsulated. In another preparation, the polymer is dissolved in an organic solvent, the “oil,” (yellow). The water-immiscible solvent is selected to be highly volatile to facilitate its removal, CH_2Cl_2 is a common choice. The protein solution and the polymer solution are mixed; since water is not miscible in the organic solvent, an emulsion forms, and the size of the watery droplets in the polymer continuous phase may be controlled via the energy used in preparing the mixture. Now what exists is a polymer solution in organic solvent, with microdroplets of protein solution in aqueous buffer dispersed throughout. How does one make microparticles from this? This emulsion is further mixed with water that contains an emulsion stabilizer (light blue), such as poly(vinyl alcohol), and again energy is added, although at a lower level so as to obtain larger droplets. Following the mixing is a watery continuous phase, with microdroplets of polymer solution in the organic solvent, which in turn have smaller droplets within them made up of aqueous buffer containing the bioactive protein—a so-called water-in-oil-in-water double emulsion, that is, an emulsion within an emulsion. This double emulsion can be subjected to low pressures to evaporate off the organic solvent, during which the polymer solution merely becomes a solid polymer, containing the watery droplets of protein throughout—microparticles of polymer with smaller microdroplets and nanodroplets of protein in buffer. This method has been used to develop a few commercial products in protein drug delivery.

**FIGURE 12.13**

Water-in-oil-in water double emulsion to generate polymer microparticles containing protein drugs.

If using hydrophobic polyesters for the polymer component, they tend to degrade, yielding acidic products, and the pH of the interior of the microparticle may become very low.^{61,62} This can lead to chemical changes in the protein, such as deamidation of glutamine and asparagine residues to glutamic acid and aspartic acid residues, respectively. Since this unfavorable side reaction is due to the acidic microenvironment within the particle, approaches have been developed to coencapsulate bases, such as $\text{Mg}(\text{OH})_2$, a common antacid.⁶³ This has been demonstrated to dramatically improve the stability of the encapsulated protein and has been shown to enhance the delivery of protein growth factors such as FGF-2 and BMP-2.

The examples in this section demonstrate that hydrophobic degradable polymers, in the form of microparticles or complete scaffolds, can be used to incorporate protein drugs and control their release. These scaffolds can be used to incorporate multiple growth factors with various release rates.

12.6 Bioactive factors bound to affinity sites within matrices

In synthetic and biomolecular scaffolds that are highly permeable, it is difficult to retain protein drugs for prolonged periods. It was mentioned earlier, in the context of gelatin matrices, for example, that low-affinity electrostatic interactions only partially remedy this. It was further mentioned that some

biomolecular complexes between growth factors and heparan sulfate proteoglycans or between growth factors and ECM proteins have an evolved **high-affinity interaction**. This evolved affinity can be further engineered to prolong sustained release, and this forms the topic of the present section.

Most growth factors of interest in tissue engineering are referred to as heparin-binding growth factors. They contain positively charged domains on their surfaces⁶⁴ that bind with a relatively high affinity to heparan sulfate-containing proteoglycans in the ECM. This affinity for heparin has been used in engineering systems for the controlled release of growth factors. Early work utilized heparin bound into hydrogel matrices, where the heparin then served to bind the growth factors. Microparticles of such bio-functional gels have been used in controlled release of FGF-2.^{65,66} Chemically modified heparin has been bound within poly(ethylene glycol) hydrogels to bind FGF-2 for use in inducing differentiation of mesenchymal stem cells.⁶⁷

Quite sophisticated delivery matrices can be developed based on this principle of heparin affinity. For example, fibrin derivatives have been constructed, which contain heparin-binding peptides.^{64,68,69} These peptides then bind heparin, which can then bind heparin-binding growth factors. The higher the ratio of bound heparin to incorporated growth factor, the more delayed is the release of the heparin-binding growth factor.⁷⁰ Although the overall interactions are a bit indirect, the approach leads to a dramatically prolonged release of many growth factors, including nerve growth factor (NGF), FGF-2, TGF- β 1, VEGF, and BMP-2.^{69,71} The control release of VEGF from a heparin functionalize alginate matrix is shown in Fig. 12.14. This demonstrates that a heparin (HP)-functionalized alginate (ALG) hydrogel prolongs the release of VEGF as compared to the hydrogel without heparin. A peptide with the RGD integrin-binding motif is also incorporated within a set of hydrogels; the addition of the peptide does not affect the release rate of VEGF.

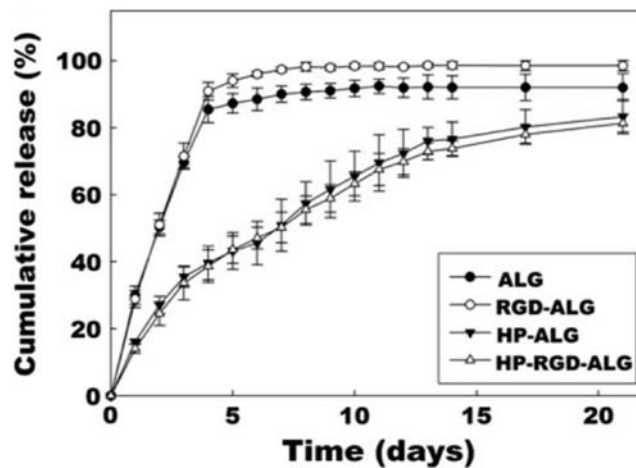


FIGURE 12.14

Heparin-functionalized alginate for control release of VEGF. From Jeon O, Powell C, Solorio LD, Krebs MD, Alsberg E. Affinity-based growth factor delivery using biodegradable, photocrosslinked heparin–alginate hydrogels. *J Contr Release* 2011;154:258–266.

Nonproteoglycan ECM proteins that do not contain highly negatively charged sugar chains, such as fibrinogen and fibronectin, can also be used to bind a number of growth factors.^{33,37,72,73} Small domains within the ECM proteins are responsible for the growth factor-binding activity and can be used instead of the full-length proteins, since the latter are often more difficult to translate into the clinic due to cost and safety reasons. For example, the main **growth factor-binding domain** of fibronectin has been recombinantly produced and incorporated into fibrin, to sequester VEGF-A165, PDGF-BB, and BMP-2.^{9,33} Similarly, the growth factor-binding domain of fibrin was incorporated within a PEG hydrogel in combination with a cell adhesion peptide. The synthetic matrix, intended to mimic fibrin, was loaded with FGF-2 and/or PlGF-2 and implanted in full thickness skin defects in a mouse presenting impaired wound healing. This scaffold, incorporating the growth factor-binding domain and growth factors, induced wound healing comparable to the one observed when the growth factors were delivered within a natural fibrin matrix.

Besides the fact that the ECM binds growth factors, the **cooperative interactions** between ECM proteins, growth factors, adhesion receptors, and growth factor receptors can be exploited.^{74–76} For instance, ECM/growth factor complexes can act as a cooperative signaling molecule and induce the formation of clusters between growth factor receptors and integrins. Because several molecules involved in the signaling machinery of growth factors and integrins are common (see [Chapter 4 Cell signaling and 8 Cell-material interactions](#)), the formation of such clusters can enhance and prolong the signaling of growth factor receptors. Exploiting the **synergistic signaling** between the growth factor receptors and adhesion receptors is particularly interesting, because it allows strong cellular signaling yet with a lower dose of growth factor. For example, a multifunctional recombinant fragment of fibronectin has been engineered to comprise both the major integrin-binding and growth factor-binding domains. This fragment was able to mediate synergistic signaling between the integrin $\alpha_5\beta_1$ and the growth factor receptors for VEGF-A165, PDGF-BB, and BMP-2. In a model of chronic wounds in diabetic mice and in a model of bone repair in rat, codelivery of low doses of growth factors with the multifunctional fibronectin fragment induces skin repair and bone regeneration using doses at which the growth factors delivered without the fragment had no significant effects.⁹

The strategies discussed earlier always involve the use of a biomaterial to control the release of growth factors. Therefore, when the growth factor is released from the biomaterial or when the biomaterial is degraded, the growth factor quickly diffuses and signals within the surrounding tissue or may enter the circulatory system. This is especially true for growth factors that do not display a strong binding to the ECM. In contrast, some growth factors have evolved to strongly bind ECM components. For example, PlGF-2 binds exceptionally strongly and promiscuously to ECM proteins, such as fibrin, fibronectin, and osteopontin, the binding is mediated by a 22-amino acid domain within PlGF-2.³⁶ By fusing this peptide sequence of PlGF-2 to VEGF-A, PDGF-BB, and BMP-2, improved ECM-binding variants have been engineered displaying 2- to 100-fold greater affinities to ECM proteins and heparan sulfate. Therefore, these ECM superaffinity growth factors could stay at the site of delivery for an extended period, and they could induce repair in rodent models of chronic wounds and bone defects that were greatly enhanced as compared to treatment with wild-type growth factors.

The examples in this section demonstrate that biologically evolved affinities between protein drugs and biomolecules can be exploited in engineered systems for sustained release, and that these affinities can be endowed upon both synthetic and biological matrices for tissue engineering.

12.7 Bioactive factors covalently bound to matrices

As mentioned in the Introduction, controlled release in tissue engineering does not always imply that the bioactive agent is released from the support; a desired controlled release outcome may mean that the bioactive molecule is retained on the surface of a polymer matrix or scaffold. This is true especially for adhesion factors as drugs but may be true as well for growth factors. We begin with the obvious case of the adhesion factor, and then proceed to the less obvious case of the growth factor, to make some cases in point.

For an adhesion factor to carry out its biological function of transmitting stress between a substrate outside the cell and the cytoskeleton within the cell, the adhesion factor must be immobilized to the substrate (see [Chapter 8 Cell-material interactions](#)). In the case of biopolymeric scaffolds such as collagen and fibrin, there exist many adhesion ligands that are built into the scaffold material by evolution. In the case of synthetic matrices, it is necessary to incorporate exogenous ligands within the scaffold to enable biospecific cell adhesion.

Several chemical approaches have been developed by which to endow materials with exogenous cell adhesion sites.^{77,78} For example, with hydrogels, adhesion peptides may be covalently coupled to an immobilization site through a linking polymer, such as a poly(ethylene glycol) chain.⁷⁹ Such approaches have been used to direct cell migration on hydrogel surfaces,⁸⁰ for example, by incorporating gradients of the adhesion peptides on the biomaterial. By carefully selecting the peptide that is incorporated within the overall nonadhesive hydrogel platform, one may select between different adhesion receptors on the surface of the cell to induce different functions. Such approaches can be used to create biomaterials that demonstrate selectivity to specific cell types, such as endothelial cells.^{81,82} One can also trigger specific cell functions with immobilized adhesion ligands, such as differentiation of precursor cells⁸³ and osteoblasts.⁸⁴

Even biopolymeric matrices that already have cell adhesion characteristics can benefit from the incorporation of additional cell adhesion domains. This is because the cell adhesion peptides and proteins do more than just provide adhesion; they also provide specific cell differentiation cues much like growth factors do. Interesting examples can be taken in the context of engineering of fibrin scaffolds. Peptides and proteins can be incorporated into fibrin during coagulation using the enzymology of coagulation. During coagulation, an enzyme, the transglutaminase factor XIIIa, links glutamine residues to lysine residues to cross-link the fibrin gel into a strong and stable matrix. It is possible to synthesize exogenous peptides or to engineer recombinant proteins incorporating a substrate site for factor XIIIa.⁸⁵ The net effect of this is that the exogenous peptide or protein will be covalently incorporated into the fibrin network during coagulation. This has been explored with adhesion peptides from the protein laminin, a protein that is involved in several morphogenetic processes, including nerve growth. When four laminin peptides were covalently coupled into fibrin matrices and the derivatized fibrin was used to repair resected nerves in the rat, the number of myelinated axons bridging the resection

site was nearly doubled compared to unmodified fibrin.⁸⁶ Fibrin is already adhesive for nerve cells, but the laminin-derived peptides bound to additional adhesion receptors and signaled greater regeneration than with the native fibrin alone. Similar effects have been seen with other adhesion proteins linked into fibrin and inducing complicated morphogenetic processes such as angiogenesis.⁸⁷

Growth factors are also interesting drugs to consider for immobilization within biomaterial matrices and on the surfaces of biomaterial scaffolds. One of the earliest *in vitro* demonstrations that substrate-bound growth factors could be biologically active was carried out with epidermal growth factor for cell differentiation.⁸⁸ One of the earliest schemes to construct injectable matrices was by derivatization of type-I collagen with the growth factor TGF- β 2. The growth factor was linked to collagen via a reactive bifunctional linker and was useful as an injectable biomaterial scaffold for cell infiltration.⁸⁹

The scheme described earlier in this section for functionalization of fibrin using the transglutaminase factor XIIIa has also been used for chemical linking of growth factors within fibrin. Growth factors are recombinantly expressed as a fusion protein to include a sequence that is coupled by factor XIIIa to fibrin, with an intervening amino acid sequence that is cleaved by cell-activated enzymes. This scheme has been carried out with VEGF-A, BMP-2, NGF, TGF- β , and IGF-I to demonstrate high activity *in vitro* and *in vivo*.^{90–94} Additionally, these substrate-bound growth factors can interact with the physical aspects of their environment, such as flow near the surface of cells.⁹⁵

12.8 Matrices used for immunomodulation

Similar to wound healing, the development of an immune response is a tightly orchestrated process involving multiple cell populations, soluble factors, and cell adhesion molecules. Likewise, tissue-engineered scaffolds for immunomodulation hold immense therapeutic potential and contribute to strategies for vaccination, cancer, autoimmunity, inflammation, and a variety of diseases stemming from dysregulated immunity. The design of such scaffolds incorporates the same methods of controlled release previously discussed for wound healing and bone regeneration, and one could argue that an even higher level of controlled delivery is necessitated. In this section, we shall discuss the up-and-coming field of **immunoengineering**, which applies tissue engineering principles to the design of materials intended to modulate the immune system.

Depending on whether an immune response is mediated by molecular or cellular mechanisms, immunity can be, respectively, divided into two categories: innate and adaptive. Adaptive immune responses require an intricate series of cellular interactions that are well suited for manipulation and guidance by synthetic microenvironments. The first step in cell-mediated immunity is the activation of professional antigen-presenting cells (APCs), which include dendritic cells, macrophages, and B cells. APC activation is dependent on the presence of immunogenic molecules, or **antigens**, as well as immunostimulatory molecules, referred to as **adjuvants**, which can be incorporated into an engineered scaffold or matrix. The mechanisms by which APCs are activated determine whether an inflammatory or tolerogenic response is elicited.

The fibrin scaffold that forms within a wound following tissue injury is a natural example of how both antigen and adjuvant can be properly displayed for an appropriate immunological response. Fibrin has also been extensively used in tissue engineering applications as described early in this chapter. It

naturally contains adhesion motifs that specifically bind to cell-surface receptors on leukocytes to enhance their infiltration into the scaffold matrix. In particular, the leukocyte $\alpha_M\beta_2$ /MAC-1 integrin receptor interacts with the peptide sequence NRLSIGE within the fibrinogen gamma chain and is critical to the induction of in vivo inflammatory responses.⁹⁶ In addition to integrin-binding motifs, key soluble proteins, that is, cytokines and chemokines, need to be present to help control the immune response. They are produced by recruited inflammatory cells or could be delivered within the hydrogel scaffold. Cytokines are factors responsible for immune cell activation, differentiation, and proliferation, while chemokines direct cell migration. As mentioned earlier, adjuvants are soluble factors that play a fundamental role in the activation of APCs. APCs possess specialized membrane-bound and intracellular toll-like receptors (TLRs) that bind and detect evolutionarily conserved adjuvants from microbes and pathogens. TLR agonists are pathogen-associated molecular patterns (PAMPS) that include lipopolysaccharides, viral and bacterial DNA and RNA, as well as synthetic small molecules such as imiquimod derivatives and monophosphoryl lipid A. Further, APCs process soluble antigenic components, such as foreign proteins and carbohydrates present within the matrix, and when properly activated by PAMPS, they can activate effector cells for the subsequent generation of antigen-specific cytotoxic and antibody responses.

Following exposure to antigen, adjuvant, cytokines, and chemokines, APCs are induced to migrate out of the scaffold into secondary lymphoid organs, which are specialized microenvironments optimized for leukocyte activation and function. Thus, immunomodulatory scaffolds must not only recruit specific cell populations, but they must also direct their exit from the scaffold for the induction of subsequent effector functions. Secondary lymphoid organs contain the effector cells of the immune system, lymphocytes, whose function is directed by the activated APCs. Lymphocytes comprise T cells and B cells, which form a unique cell–cell junction with activated APCs referred to as the immunological synapse. The types and concentrations of cytokines and chemokines present during formation of the immunological synapse can drastically change the induced cellular responses. Thus, controlled delivery of soluble factors is again a critical design criterion. Further, the formation of the immunological synapse is enhanced by cell adhesion motifs and the 3D architecture of lymphoid organs. The supportive stromal cells that comprise the connective tissues within lymphoid organs are specialized to express chemokines and cell adhesion ligands that, respectively, attract specific leukocytes and promote their adhesion within separate domains. The lymph node, for example, is divided into B and T cell zones that greatly enhance lymphocyte interactions with APCs and promote the generation of antibodies and cytotoxic T cells that are precisely tailored to address a particular infection as necessary. A detailed description of the immunological processes that induce these responses is beyond the scope of this text, but we intend to call attention to the lessons that can be learned from natural microenvironments when designing immunomodulatory tissue engineering constructs.

As described earlier, in vivo microenvironments have evolved to present immunostimulatory molecules to immune cells as well as to encourage essential leukocyte cell–cell interactions, and thus, tissue-engineered immunomodulatory scaffolds are frequently designed to mimic the natural microenvironments of fibrin matrices and secondary lymphoid organs. Key design parameters to consider include the selection and rate of delivery of multiple cytokines and chemokines, the type and surface concentration of cell adhesion molecules, and the overall 3D structure of the microenvironment. Most therapeutic scaffolds have therefore been composed of either PLGA, which is advantageous for controlled delivery

of soluble factors, or natural fibrinogen or collagen matrices that contain important cell adhesion motifs. For example, PLGA scaffolds that contain synthetic TLR agonists, cancer antigens, and the chemokine GM-CSF that attracts dendritic cells have been developed.⁹⁷ The scaffold could thus recruit dendritic cells via a chemokine gradient and stimulate them with cancer antigens in the presence of a potent adjuvant. The dendritic cells were thus induced to leave the scaffold and elicit tumor-specific lymphocyte responses within secondary lymphoid organs. A PLGA scaffold vaccine also known as WDVAX, which contains clinical grade GM-CSF, autologous tumor cell lysate, and CpG-ODN (a TLR agonist), is currently undergoing a phase I clinical trial (NCT01753089) for the treatment of Stage IV metastatic melanoma. The PLGA scaffolds described above require a surgical incision for the implantation procedure; therefore, easily injectable systems have been developed as alternatives. A good example of an injectable system are **cryogels**, which are usually comprised of polymers such as gelatin, alginate, PEG, or hyaluronic acid. A macroporous cryogel scaffold formed via cross-linking of methacrylated-PEG and methacrylated-alginate was able to deliver GM-CSF, CpG-ODN, and multiple myeloid leukemia antigens efficiently *in vivo*.¹⁰³ These scaffolds successfully infiltrated dendritic cells and sustained their activation in an immunosuppressive environment of an acute myeloid leukemia animal model, subsequently reducing the tumor burden.

Due to their highly efficient recruitment of immune cells such as DCs, T cells, B cells, and monocytes, biomaterial scaffolds are commonly used as artificial biomimetic lymphoid structures. An injectable alginate hydrogel that contained preactivated dendritic cells has been developed.⁹⁸ After injection, T cells were recruited to this artificial “node” and were directed to perform effector functions after interacting with the activated APCs. Several attempts have been made to better mimic the 3D structures of secondary lymphoid organs to more easily control or model elicited immune responses. A bioreactor containing scaffolds composed of macroporous cellulose microspheres has also been developed.⁹⁹ By culturing primary human tonsil cells within this *in vitro* system, T cells and B cells were found to be enriched in separate zones and antigen-specific antibody responses could be generated. Advanced B cell follicle organoids mimicking the microenvironment of lymphoid tissues was engineered using gelatin hydrogels for an accelerated germinal center (GC) reaction by providing cell–cell signals to naïve B cells.^{104,105} In this approach, RGD-conjugated gelatin hydrogel scaffolds were reinforced with silicate nanoparticles and encapsulated with fibroblasts expressing B cell survival and activation cues (BAFF and CD40L) alongside soluble IL-4. These 3D scaffolds served as ECM support to the cultured naïve B cells and allowed primary B cell proliferation and differentiation to the GC phenotype with isotype class switching. Recently, efforts have been made toward the development of microfluidic-based lymph node on-chip technologies. For example, a two-compartment chip model has been employed consisting of T cells and B cells seeded into a Matrigel-collagen gel in one compartment with media flow in another compartment.¹⁰⁶ In this model, depending on the fluid flow, lymphocytes were able to assemble after 4 days of culture. Interestingly, B cell class switching (production of IgG) and CD138+ plasma cell formation was also observed upon stimulation with IL-4 and anti-CD40 or dead bacteria.

In vivo generation of lymphoid tissue has also been achieved. Implantable collagenous “sponge-like” scaffolds were seeded with lymphoid stromal cells which generated lymphoid-tissue-like organoids that could elicit strong antibody responses (Fig. 12.15A).¹⁰⁰ The incorporation of both lymphoid stromal cells as well as activated dendritic cells was found to be essential for the clustering of T cells, stained

using Thy1.2 in red, and B cells, stained using B220 in green, into separate zones within the secondary lymphoid organ-mimicking structures (Fig. 12.15B).

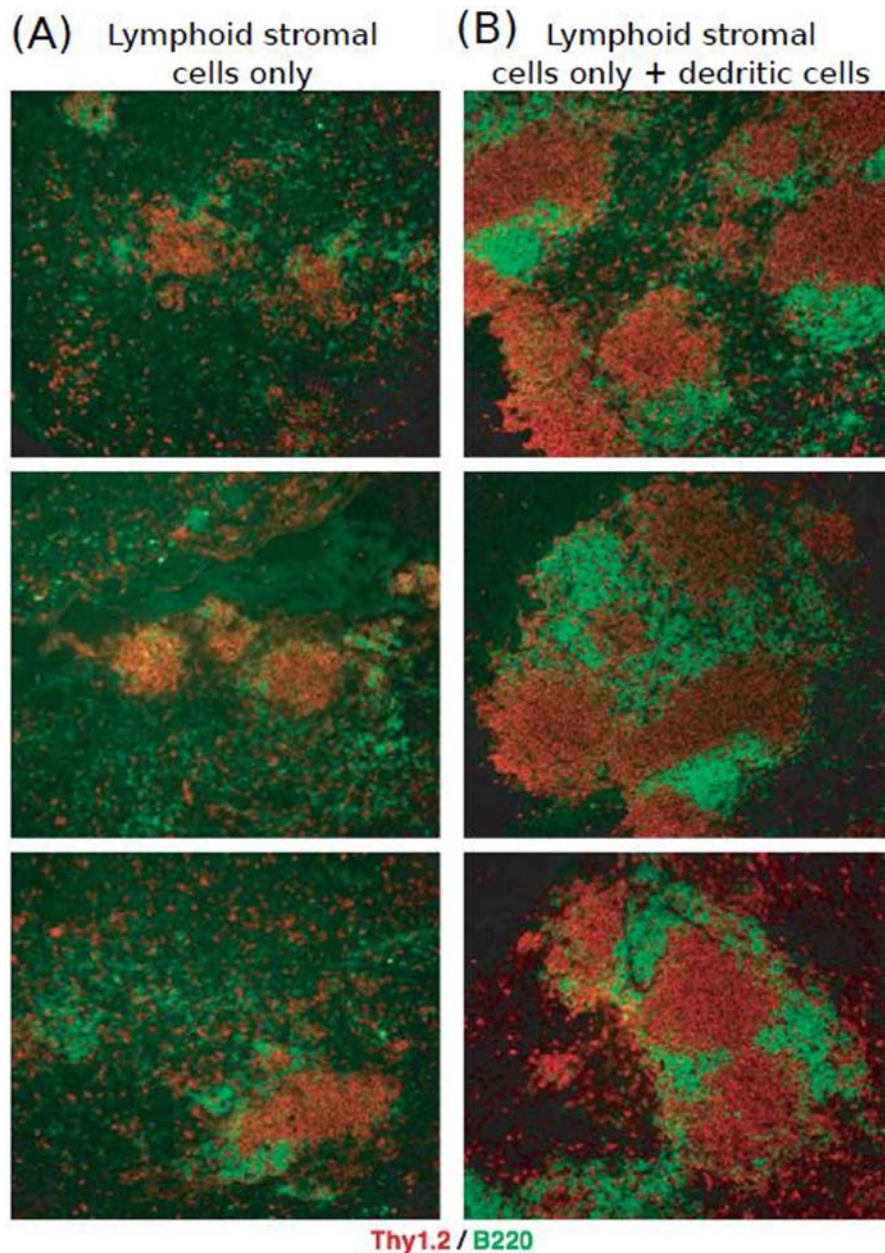


FIGURE 12.15

Inclusion of activated dendritic cells (DCs) in collagenous scaffold. From Suematsu S, Watanabe T. Generation of a synthetic lymphoid tissue-like organoid in mice. *Nat Biotechnol* 2004;22:1539–1545.

Biomaterial scaffolds have also been studied as adoptive cell transfer vehicles for tissue engineering applications. For instance, a biopolymer scaffold made up of alginate and a cell adhesion molecule which acts as a collagen-mimetic peptide, has been developed to deliver tumor-specific T cells. After implantation, these scaffolds enhanced the viability and expansion of T cells and allowed their migration with a velocity comparable to that found in lymphoid organs.¹⁰⁷ Likewise, chimeric antigenic receptors (CAR T cells) and stimulator of IFN genes (STING) that were incorporated into alginate scaffolds induced superior antitumor immune responses in immunosuppressive mouse models of pancreatic cancer and melanoma.¹⁰⁸

In this section, we have seen that tissue-engineered constructs can be used for immunomodulation by applying methods of controlled release that were initially developed for tissue healing and regeneration. Tailoring matrices to present antigens, adjuvants, cytokines, and chemokines in a fashion that mimics natural microenvironments can allow precise control over cellular migration and the immune response.

Summary

- Most drugs of interest for controlled release in tissue engineering are proteins and peptides. These include adhesion factors and growth factors. The hydrophilic nature of these drugs imposes many constraints on the controlled release strategies that can be employed.
- In tissue engineering, controlled release sometimes means that the drug is released from the surface or bulk of an implant slowly over long periods of time, and sometimes, it means that the drug is immobilized on the surface of or throughout the material and is not released at all. The difference depends on the mode of action of the drug. For example, adhesion factors are only active when immobilized.
- Hydrophilic drugs may be incorporated homogeneously through hydrophilic matrices, but only heterogeneously through hydrophobic matrices. In hydrophobic matrices, they may be incorporated as solid drug particles through solvent processes, or as aqueous pools rich in protein, for example, through water-in-oil-in-water double emulsion processes.
- Protein drugs may be released from hydrophilic matrices through diffusive release or through bulk degradation, or from hydrophobic matrices through surface degradation. With diffusive release, it is not possible to obtain constant releases rates over time. With bulk degradation, this can be obtained if certain features of the network structure are appropriately balanced. It is easier to achieve this with surface degradation.
- Many growth factors bind to other biological molecules, such as heparin and heparan sulfate proteoglycan. This affinity can be exploited in heparin-containing systems for controlled release. Some growth factors have evolved affinities for ECM proteins, such as fibrin, fibronectin, and collagen. This can also be exploited in controlled release.
- Hydrogels resemble 3D fishing nets, and protein drugs can be efficiently entrapped within hydrogels if the distance between cross-links within the network is sufficiently close to the diameter of the protein being released.
- Degradable polyesters, such as poly(lactic acid), poly(glycolic acid), poly(ϵ -caprolactone), and their copolymers are useful in controlled release from tissue engineering scaffolds.
- The internal pH in degradable polyesters is often very acidic, which may cause problems in the long-term stability of protein drugs being released. This can be corrected by coencapsulating pharmaceutically acceptable bases, such as $\text{Mg}(\text{OH})_2$.
- The creation of tissue-engineered constructs will play an increasing role in immunomodulation by allowing for cell migration into environments tailored with antigens, adjuvants, cytokines, and chemokines.

Classical experiment

Controlled protein release from hydrophobic polymers

In the 1970s, many investigators were working in the delivery of hydrophobic drugs from hydrophobic materials; however, very few investigators were working on the controlled release of protein drugs. In a seminal paper in 1976 in *Nature*,¹⁰¹ this limitation was decisively broken. Robert Langer, now a professor and world-renowned researcher at the Massachusetts Institute of Technology and then a postdoctoral fellow working with Prof. Judah Folkman at Harvard Medical School, shared his results on controlled release of proteins from hydrophobic polymers, both in vitro and in vivo. The method was simple and elegant and has been used by countless researchers around the world and in numerous clinical products to date. The method takes advantage of the fact that many polymers are soluble in organic solvents, but proteins are generally not. If a protein is dissolved in a buffer, and then a water-miscible organic solvent (such as ethanol) is mixed into the water; denaturation of the protein typically occurs and perhaps permanently loses some or all of its biological activity. However, if the protein is mixed

as a solid particle directly into an organic solvent, it cannot be denatured, because it has no molecular mobility in the solid state. In this method, protein particles are mixed into an organic solution of a hydrophobic polymer, such as ethylene–vinylacetate copolymer in CH_2Cl_2 . The solvent is then evaporated to form a polymeric film or some other form. If the protein particles within the film are sparse and are surrounded by the polymer, then little protein release occurs. However, if the protein particles are denser and contact each other, then a slowly dissolving tortuous path of protein may exist within the polymer, allowing access of water outside the sample to the protein-rich interstices within the polymer. This in turn allows slow release. Release of proteins up to 100 days was observed, and the rate of release depended on the molecular weight, and thus the diffusion coefficient, of the test protein, as illustrated in Fig. 12.16. To test this concept in vivo, Langer encapsulated an angiogenic protein within ethylene–vinylacetate pellets and implanted these in the corneas of rabbits, a tissue in which vessels can be easily visualized. The proteins released from these pellets-induced angiogenesis from

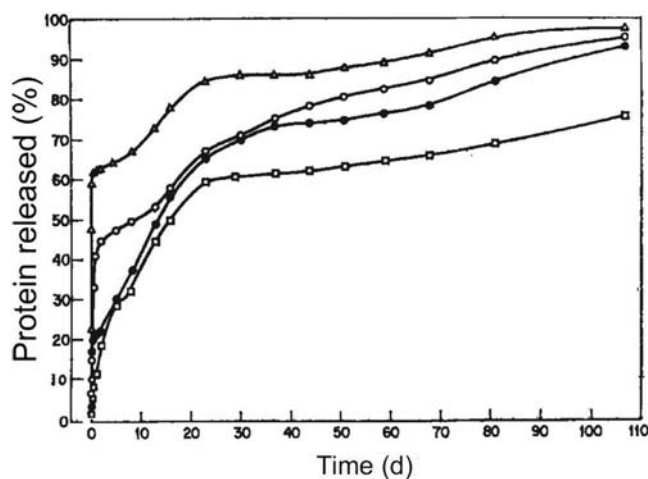


FIGURE 12.16

The cumulative protein release over time, as demonstrated first by Langer and Folkman. From Langer R, Folkman J. *Polymers for sustained-release of proteins and other macromolecules*. *Nature* 1976;263:797–800.

Classical experiment—continued

the sclera into the cornea in a manner that was dependent on the protein being released. The method is easy to carry out and to scale into an industrial process, which has led to its success.

Proteins released from ethylene vinylacetate copolymer demonstrated that long-term release could be obtained

and that the rate of release depended upon the molecular weight, and thus the diffusion coefficient, of the probe protein. Lysozyme (14 kDa, triangle), soybean trypsin inhibitor (21 kDa, open circle), alkaline phosphatase (88 kDa, closed circle), and catalase (250 kDa, square) were used.

State-of-the-art experiment

Immobilization and cell-directed controlled release of engineered protein-based therapeutics for regenerative medicine applications

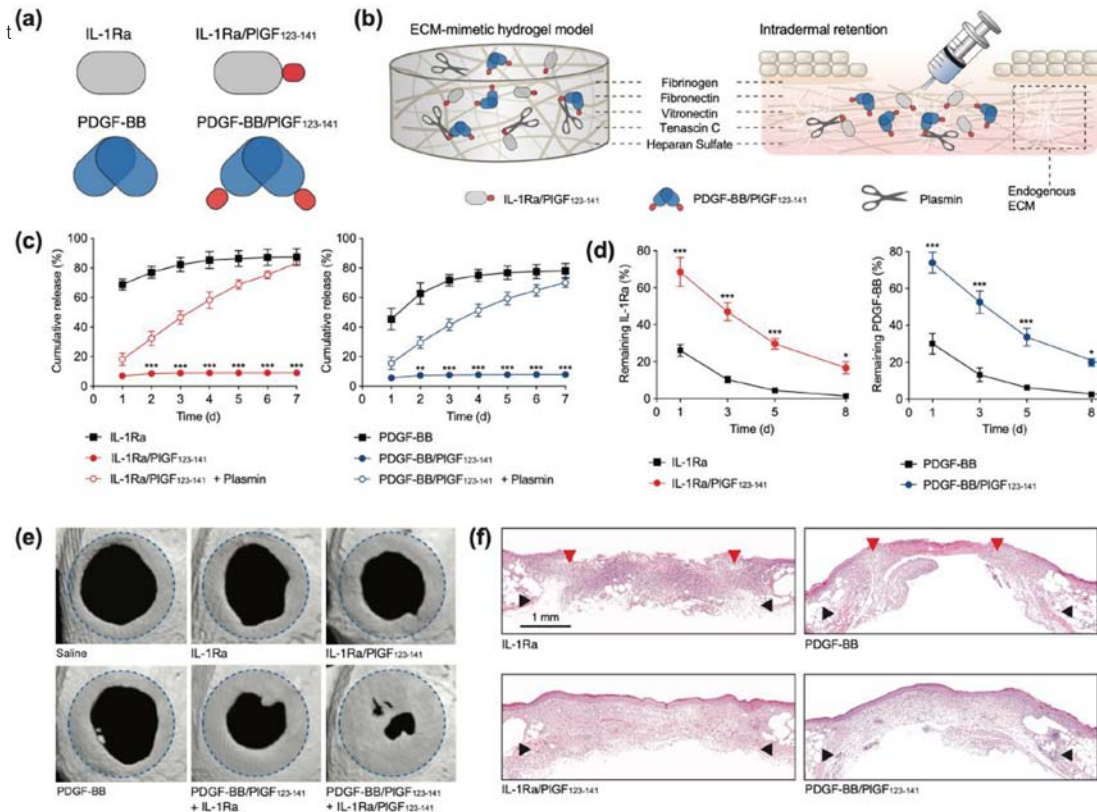
Without an optimal delivery system, important biologics used in regenerative medicine such as cytokines and growth factors need to be delivered at very high doses to have a significant therapeutic effect. High doses are required because of the relatively short half-life of these types of proteins and their very rapid diffusion to other parts of the body. However, high doses of cytokines and growth factors may lead to potentially dangerous side effects. For instance, the growth factor PDGF-BB might trigger the development of an undetected tumor somewhere in the body, because many cells in the tumor microenvironment express receptors for PDGF-BB. As another example, high levels of the cytokine inhibitor interleukin-1 receptor antagonist (IL-1Ra) might reduce the ability of mounting an immune response to fight infections, because interleukin-1 is a critical cytokine in immunity. Therefore, an optimal delivery system has been developed to use low doses of PDGF-BB and IL-1Ra therapeutics, and to ensure their precise localization at the delivery site. Recombinant variants of PDGF-BB and IL-1Ra have been engineered to process a 'built-in' delivery system by conferring them a drastically increased affinity for ECM components present in tissues (e.g., ECM proteins, heparan sulfate). Specifically, the therapeutics were engineered to include a sequence derived from placenta growth factor, PIGF₁₂₃₋₁₄₁, that binds exceptionally strongly and promiscuously to ECM components. For instance, insertion of PIGF₁₂₃₋₁₄₁ in PDGF-BB and IL-1Ra conferred

superaffinity to a hydrogel made of ECM proteins and to ECM components naturally present tissues (e.g., in skin and bone). While the factors displayed very high affinity to ECM proteins, they could be released from the ECM-based hydrogel via the protease plasmin (a protease usually present in injured tissue), since PIGF₁₂₃₋₁₄₁ naturally contains cleavage sites for numerous proteases. Thus, following the delivery of the factors into an injured tissue, cells that migrate into the tissue encounter a field of hydrogel- or ECM-bound PDGF-BB and IL-1Ra, which can be liberated by migrating cells through local proteolytic remodeling. This strategy demonstrated in animal models that low doses of engineered PDGF-BB and IL-1Ra therapeutics can be very potent to regenerate bone and to trigger skin wound healing.

Within Fig. 12.17, Fig. 12.17A shows the PIGF₁₂₃₋₁₄₁ sequence (in red, ECM-binding sequence) is fused to the C-terminus of PDGF-BB (in blue, naturally a homodimer protein) and IL-1Ra (in gray, naturally a monomeric protein), in order to generate PDGF-BB/PIGF₁₂₃₋₁₄₁ and IL-1Ra/PIGF₁₂₃₋₁₄₁. Figs. 12.17B–D depict the retention of PDGF-BB and IL-1Ra variants in an ECM-based hydrogel and in skin following intradermal injection (PDGF-BB and IL-1Ra variants are detected using enzyme-linked immunosorbent assays). The graphs Fig. 12.17C show the amount of PDGF-BB and IL-1Ra variants released from the hydrogel into a physiological saline solution (replaced every day). PDGF-BB and IL-1Ra were quickly released, while PDGF-BB/PIGF₁₂₃₋₁₄₁ and IL-1Ra/PIGF₁₂₃₋₁₄₁ remained sequestered in the hydrogel. Addition of plasmin (represented as scissors) in the saline solution triggered

Continued

State-of-the-art experiment—continued

**FIGURE 12.17**

Important steps to tissue restoration in diabetic wounds using IL-1 receptor antagonist. From Tan JL, et al. *Restoration of the healing microenvironment in diabetic wounds with matrix-binding IL-1 receptor antagonist*. Commun Biol 2021;4(1):1–11 and Julier Z, et al. *Enhancing the regenerative effectiveness of growth factors by local inhibition of interleukin-1 receptor signaling*. Sci Adv 2020;6(24):eaba7602.

because ECM proteins and PIGF₁₂₃₋₁₄₁ are cleaved by plasmin. The graphs in Fig. 12.17D show that wild-type PDGF-BB and IL-1Ra disappeared rapidly from the injection site in mouse skin, while the ECM-binding PDGF-BB and IL-1Ra stayed for an extended period. In Figs. 12.17E and 12.17F low doses of PDGF-BB/PIGF₁₂₃₋₁₄₁ and IL-1Ra/PIGF₁₂₃₋₁₄₁ are demonstrated to induce bone regeneration and skin wound closure in mice. Micro-CT reconstitution of cranial bone defects treated with PDGF-BB and IL-

1Ra/PIGF variants delivered via a fibrin hydrogel are shown in Fig. 12.17E (1 month time point, original defect is indicated with a blue dashed line). Histology sections of diabetic wounds treated with PDGF-BB or IL-1Ra variants delivered topically in a saline solution are shown in Fig. 12.17F (9-day time point, hematoxylin and eosin staining, black arrows indicate wound edges, red arrows indicate where the epithelium stops).

12.9 Recommended literature

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12.10 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
1. What is meant by the term “therapeutic window”?
 - A. The time over which a drug is released.
 - B. The concentration range between the effective drug concentration and a toxic drug concentration.
 - C. The spatial region around a depot in which drug is present at an effective concentration.
 2. What is meant by the term “solid solution”?
 - A. A drug distributed in a material depot as solid particles.
 - B. A drug distributed in a material homogeneously mixed with the material.
 - C. A drug that is soluble in water, distributed in a material before release.
 3. By what mechanism would a material like a collagen sponge degrade in vivo?
 - A. By bulk enzymatic hydrolysis.
 - B. By surface-mediated hydrolysis.
 - C. By cell-mediated enzymatic hydrolysis.
 4. By what mechanism would a material like poly(ϵ -caprolactone) degrade in vivo?
 - A. By bulk hydrolysis.
 - B. By bulk enzymatic hydrolysis.
 - C. By surface-mediated hydrolysis.

5. In order to obtain a material that degrades by surface erosion, what physicochemical characteristics should the material have?
 - A. The material should be hydrophobic.
 - B. The material should be hydrophilic.
 - C. The material should be soluble in water.
 6. When a material degrades by enzymatic action, what determines whether erosion is surface-mediated or bulk?
 - A. The source of the enzyme, namely present in body fluids or associated to the cell.
 - B. The diffusion coefficient of the enzyme in the material.
 - C. Both A and B.
 7. What kinds of molecules are most morphogenetic compounds?
 - A. Peptides and proteins.
 - B. DNA
 - C. Both A and B.
 8. What is meant by protein denaturation?
 - A. When a protein partially unfolds and loses its activity.
 - B. When a protein is too dilute to be active.
 - C. When a protein adsorbs to a material surface.
 9. Some classes of morphogenetic molecules must be immobilized to be bioactive. What class(es) are these?
 - A. Growth factors.
 - B. Adhesion factors.
 - C. Both A and B.
 10. Would release of a protein morphogen like BMP-2 from a proteinaceous material like a fibrin gel be expected to be linear?
 - A. Yes.
 - B. No.
 - C. It depends on cellular activity within the gel.
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations.
1. In your own words, explain the importance of controlling the release of biological factors from matrices.
 2. Briefly describe the advantages and disadvantages of physical entrapment versus covalent immobilization of biological factors within matrices.
 3. Briefly describe the advantages and disadvantages of specific versus nonspecific affinity interactions to control the release of biological factors within matrices.
 4. Explain two roles fibronectin can play in hydrogels for wound healing.
 5. Explain the importance of proteolytic degradable linkers in matrices for wound healing.

Challenge-based learning

Controlled release of bioactive molecules to tune the healing of large bone defects

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Bone defects due to trauma, congenital defects, diseases, and aging are a serious threat to human health. A complex but well-orchestrated fracture healing cascade is triggered when bone fractures. This process includes an acute inflammatory response, the release of specific biomolecules at the injury site, and the recruitment of cells to generate bone callus. To facilitate the spontaneous biological healing cascade, both ends of the fracture need to be in close proximity. If the gap is too big, fractures do not heal adequately. Therefore, critical-sized bone defects (CSBDs) are treated in the clinic with autologous bone. Autologous bone grafts are the current gold standard but are limited by the volume that can be harvested from the donor and involve an invasive harvesting procedure. Allografts have limited clinical application mainly due to donor shortage. Additionally, the extracellular matrix and growth factors can be degraded or leached out during processing and sterilization steps, making allografts less biologically active. The long-term goal of this research field is to generate materials with osteo-induction capacity similar to autologous bone grafts.

Motivation and stakeholders

Osteo-induction requires the recruitment of progenitor cells and the stimulation of these cells to differentiate into bone-forming cells. During bone healing after a fracture, most of the repair is dependent on osteo-induction. Thus, there is a clinical need to develop biomaterials that can trigger the regenerative machinery of bone and modulate the spontaneous healing cascade to fix CSBDs. Solutions to mitigate this problem should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as patients with CSBDs, orthopedic surgeons, bone biologist, and biomaterial engineers.

Problem definition

Currently, bone grafts do not actively respond to the local regenerating tissue nor sense the stage of the healing cascade. Therefore, there is a lack of dynamic cross-talk between the tissue and the graft. Smart bone grafting

would require materials which can sense the stages of the healing cascade, and respond by down- or upregulating inflammation, recruitment of stem cells, inducing cartilaginous bony callus revascularization, or deposition of mineral matrix.

Challenge

To design a biomaterial that responds to one or several stages of the bone healing cascade to release bioactive molecules in a triggered and controlled manner to effectively treat CSBDs.

Learning scaffold

Reading the Controlled Release and Bone Tissue Engineering chapters and related literature will help you to understand the following:

1. The different ways to release bioactive signals from biomaterials.
2. Bone structure (lamellar vs. cancellous) and bone metabolism.
3. The bone healing cascade and direct versus indirect fracture healing.
4. The bioactive signals (molecules, growth factors, cytokines) that can accelerate bone deposition and healing.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

5. Clinical and bioengineered strategies to heal bone critical-sized defects.
6. Strategies to release bioactive signals in the fracture niche to promote bone formation and treatment of critical-sized defects.
7. Natural and synthetic biomaterials to accelerate fracture healing and the treatment of critical-sized defects.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

12.11 Glossary

Adjuvant is a substance that enhances the immune system's response to the presence of an antigen.

Antigen is a molecule or molecular structure that can bind to a specific antibody or T cell receptor. The presence of antigens in the body may trigger an immune response.

Autograft is a transplanted tissue from the same individual.

Biomolecule immobilization is a process of attaching a bioactive molecule to the surface of a biomaterial.

Block copolymer comprises two or more homopolymer subunits linked by covalent bonds.

Chemokines are a family of signaling proteins secreted by cells that induce directional movement of leukocytes, as well as other cell types, including endothelial and epithelial cells.

Closed and controlled herds are a large group of animals without contact with other animals, controlled by humans.

Composite polymer systems are multiphase materials in which biomolecules are integrated within a polymer matrix, resulting in synergistic biomechanical properties that cannot be achieved from either component alone.

Controlled release refers to the concept of drug release from a source over a sustained period at a nearly constant rate.

Cooperative interactions refer to interaction of amino acid residues in a protein where the effects of chemical or physical perturbations to any given residue are propagated to other residues by an intricate network of interactions.

Cryogels are a type of hydrogel fabricated at subzero temperatures resulting in a biomaterial with a macroporous network.

Cytokines are a broad and loose category of small proteins ($\sim 5\text{--}20$ kDa) important in cell signaling.

Denaturation is a process in which proteins or nucleic acids lose the quaternary structure, tertiary structure, and secondary structure which is present in their native state.

Diffusion coefficient is a proportionality constant between the molar flux due to molecular diffusion and the gradient in the concentration of the molecular species.

Diffusion-controlled release is the most common method of release of molecules to the surrounding environment by a process of simple diffusion.

Extracellular domains are part of a receptor protein that resides outside of the resident membrane, mainly used for interaction with other proteins.

Glass transition temperature is the temperature range at which increased molecular mobility in a polymer results in changes in thermal properties, the polymer changes from hard, solid, glassy state to a soft, not melted state.

Glycosylated refers to the state in which a carbohydrate (glycosyl donor) is attached to a hydroxyl or other functional group of another molecule (a glycosyl acceptor) in order to form a glycoconjugate.

Growth factor-binding domain is the region contained in proteins of the extracellular matrix that binds growth factors to regulate various biochemical processes in its microenvironment.

Growth factors are naturally occurring substances capable of stimulating cell proliferation, wound healing, and occasionally cellular differentiation.

High-affinity interaction are molecular interactions where a relatively low concentration of an interacting molecule is adequate to maximally occupy its binding site.

Homogeneous degradation is a process where degradation occurs throughout the material and the drug release ensues from the entire material.

Hydrogels are cross-linked hydrophilic polymers that do not dissolve in water. They are highly absorbent yet maintain well-defined structures.

Hydrolysis is any chemical reaction in which a molecule of water breaks one or more chemical bonds.

Immunoengineering is an interdisciplinary area that integrates engineering tools and principles of immunology to harnesses the power of the immune system.

Immunogenic is the property of a foreign substance to induce an immune response in the body.

Isoforms are two or more functionally similar proteins that have a similar but not identical amino acid sequence.

Lyophilization is a process in which water is removed from a product after it is frozen and placed in vacuum, allowing the ice to change directly from solid to vapor without passing through a liquid phase.

Macroporous is a material containing pores larger than $50\text{ }\mu\text{m}$.

Matrix is a nanoporous, continuous material used to provide support in a scaffold.

Micronizing is reducing the average diameter of a solid particle by milling or grinding

- Molecular mobility** is the ability of chains from one microparticle of a polymer to diffuse into the surface of neighboring microparticles.
- Morphogens** are substances whose nonuniform distribution governs the pattern of tissue development in the process of morphogenesis or pattern formation.
- Nanoporous** materials exhibit pore diameters of 100 nm (nm) or smaller.
- Pancarpal Arthrodesis** is a surgical procedure to induce fusion of the carpus (cluster of bones in the wrist between radius and ulna).
- Pore size** is the distance between two opposite walls of a pore.
- RGD** is the tripeptide that consists of arginine (R), glycine (G), and aspartate (D) and is the amino acid sequence within many extracellular matrix proteins that mediates cell attachment.
- Scaffold** is a material that has been engineered to cause desirable cellular interactions to contribute to the formation of new functional tissues for medical purpose.
- Solid solution** is a family of materials which have a range of compositions (e.g., A_xB_{1-x}) and a single crystal structure.
- Spatiotemporal delivery** is a precision delivery system that controls both the quantity/amount and time of the release.
- Spinal fusion cages** are made of metal, used to aid bone fusion in a variety of spine disorders.
- Splice variants** are mRNA transcripts produced as a result of joining the exons of a gene of different combinations.
- Supraphysiological (concentration)** refers to a substance in concentrations higher than normally found in the body.
- Surface degradation** is a process where degradation of the material occurs only at the surface and the rate of degradation is proportional to the surface area.
- Synergistic signaling** is a form of cell signaling where the effect of a combined signal is greater than the sum of separate cell signals.
- Systemic** is relating to or affecting the whole body.
- Therapeutic window** is a quantitative concentration range of the relative safety of a drug in the body.
- Tibial nonunions** are fractures that will not unite without additional surgical or nonsurgical intervention (usually by 6–9 months).
- Transmembrane receptors** are proteins at the cell membrane, containing an intracellular and extracellular domain for signal acquisition and transduction.
- Zero-order release** is a release system in which a drug is released at a constant rate, independent of the concentration of the drug.

Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Bioreactors: enabling technologies for research and manufacturing

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13.1 Learning objectives

After reading this chapter you will be able to:

- Describe general components of a bioreactor and a general bioreactor set-up.
- Identify crucial culture parameters that influence cellular behavior during bioreactor culture.
- Explain why it is necessary to mimic physiologic conditions and how they can be implemented by bioreactor technology.
- List different groups of bioreactor systems for cell expansion, production of cell-based products, and tissue engineering applications.
- Choose suitable bioreactors and monitoring systems for a specific expansion or tissue engineering process.

By enabling reproducible and controlled changes of specific environmental factors, bioreactor systems provide both the technological means to reveal fundamental mechanisms of cell function in a 3D environment, and the potential to improve the quality of engineered tissues.

I. Martin, 2004

Bioreactors are the key to translate ... tissue-based constructs into large-scale biological products that are clinically effective, safe and financially pliable.

J. Zhao, 2016

13.2 Introduction

Traditionally, the development and establishment of production processes for biopharmaceuticals is considered as bioprocess engineering and encompasses all considerations regarding the up- and downstream aspects of a **bioprocess**. In this context, bioreactors are a central element as they assign the culture parameters and allow for a tight process control and therefore highly reproducible processes and products. From this perspective, the engineering of a tissue is likewise understood as a bioprocess for the production of a specific 3D cell-scaffold construct. To produce tissue engineered cell-scaffold constructs for

in vitro models or in vivo applications, it is central to gain sufficient control on this process. This includes monitoring and control over all relevant culture parameters and preferably a high degree of automation.

In pharmaceutical bioprocesses, bioreactors are technical devices for the controlled aseptic culture of cells and manufacturing of the product. In this context, a bioreactor represents a **closed system**, a culture vessel (i.e., tank, bottle, chamber, etc.) with a medium inlet for nutrient supply and optionally a medium outlet for waste removal. During bioreactor development, there is already a high tendency toward up-scalability to be considered for effective mass production. The bioreactor is equipped with sensors to monitor **culture parameters** (i.e., temperature, CO₂, O₂, pH, etc.) and the necessary hardware and control systems to keep these parameters stable within feedback loops (heating, gas mixer, etc.). However, bioreactors for tissue engineering are thought to represent a simplification of the in vivo conditions of the target tissue. Thus, it appears that bioreactors for tissue engineering are clearly different from traditional pharmaceutical bioreactors in their appearance and functionality.

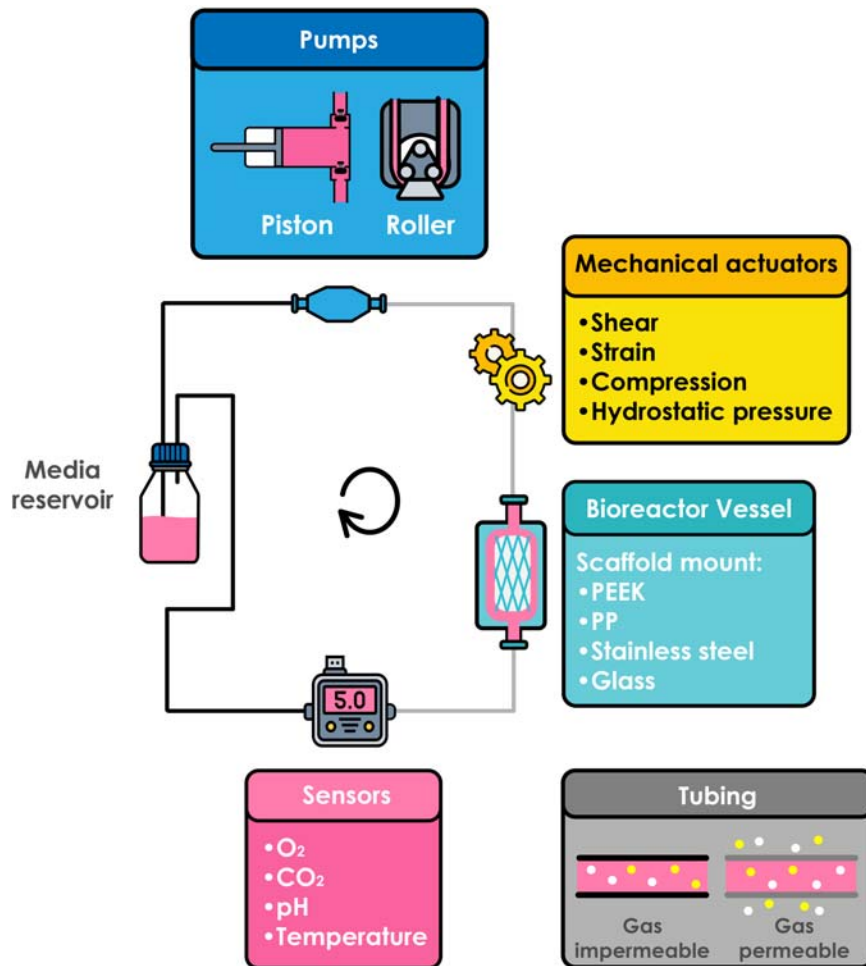
The main difference is that tissue engineering devices are designed to host a scaffold with cells mostly focusing on the representation of in vivo conditions instead of scalability and high throughput. Further, tissue engineering bioreactors comprise mechanical actuators that allow for the transduction of mechanical forces (shear, compression, strain, pressure) to the cells which support the maturation of a functional tissue. Compared to pharmaceutical bioreactors, this often results in rather complex bioreactor set-ups. Early systems filled an entire cell culture incubator, hosting only a single cell-scaffold construct.

In this chapter, we will give an overview on the basic components necessary to design a tissue engineering bioreactor, basic bioreactor set-ups, and introduce relevant sensors which will be necessary to monitor the bioprocess. Further, we will consider mass transport and physical stimuli to mimic physiologic culture conditions in a bioreactor system. Finally, we will give an overview on bioreactors for cell expansion and production of cell-based products and bioreactors for the controlled differentiation of cells.

13.3 Basic requirements

A multitude of bioreactors with various functionalities have been developed which makes it impossible to cover all possible set-ups and components. However, in this section we describe the minimal configuration that many tissue engineering bioreactors share to highlight the basic requirements. In general, bioreactors consist of a vessel, including a mount for the cell-scaffold construct. This vessel is connected to a medium reservoir and waste container via tubing and pumps. In many applications, a waste container is omitted and the medium is circulated from and to the medium reservoir (Fig. 13.1). Further, the bioreactor can be equipped with sensors. The entire set-up is operated in an incubator to ensure constant temperature and atmosphere. Although the incubator itself is not considered as a bioreactor component, it contributes to achieving vital culture conditions.

Since there is no common approach for the bioreactor chamber and the scaffold mount to fit all types of tissues, the material, the tissue architecture, and the geometry have to be adapted to the specific needs. Individual aspects of cell and developmental biology as well as geometrical functionalities of most tissues demand an individual set-up of physical stimuli. However, all components that are in contact with cells and/or the medium, especially the scaffold mount and the bioreactor vessel, must be inert,

**FIGURE 13.1**

Circular bioreactor set-up with basic bioreactor components.

noncytotoxic, and preferentially **biocompatible**. If the bioreactors are supposed to be reusable, these materials must further be repeatedly sterilizable. Steam sterilizable materials which are often used are stainless steel, glass, polyether ether ketone or polypropylene. If other methods for sterilization are available (UV-exposure, gamma-irradiation, gas sterilization), virtually all biocompatible materials might be used. Further, 3D printing for rapid prototyping of bioreactors is becoming popular and some biocompatible resins are already available. The shape of bioreactor vessels and scaffold mounts varies extremely depending on the targeted tissue and the scaffold material and size. Thus, countless variations exist which cannot be covered here. Instead, we refer to Chapter 11. The vessel itself is often equipped with mechanical actuators such as valves or linear motors to translate mechanical forces to the cell-scaffold construct.

Tubing is a crucial part of every bioreactor as it transports the media to and away from the cells. As the culture system should not be affected by unwanted external factors, the tubing must be inert and biocompatible. However, repeated steam sterilization or the use of harsh bases or acids can promote the transfer of leachables from the tubing material into the media with unknown effects on the process. Further, tubing is available from gas permeable materials such as widely used silicone tubing or gas impermeable, such as fluoroelastomers. Thus, the tubing material must be chosen carefully according to the respective bioprocess, cleaning procedures, and sterilization method.

As pumps transport the media through tubing, they are an integral part of a bioreactor system. Pumps for a bioreactor system are necessary to apply a constant or pulsatile flow and must operate at a high precision for several weeks. Peristaltic pumps, also called roller pumps, are the most widely used devices. The tubing is mounted on a rotor with several rollers and the liquid is displaced by rollers through a rotary motion. However, any free-floating cells are squeezed by the rollers which might lead to unwanted loss of cells or other side effects. Thus, piston pumps represent a viable option as any floating components experience less shear forces.

The bioreactor vessel with media reservoir and tubing is placed in an incubator with stable temperature and atmosphere. To ensure constant pH over the culture process, media contain buffer systems that require supply of 5% CO₂ to function properly. Further, in some differentiation processes, an oxygen-reduced atmosphere is necessary. For this, special incubators are available that can reduce the oxygen concentration by adding nitrogen to the atmosphere. Even more advanced incubators are available which are equipped with pumps, sensors, feedback control, documentation systems, and even mechanical actuators for transduction of mechanical forces to the cells (Fig. 13.2).

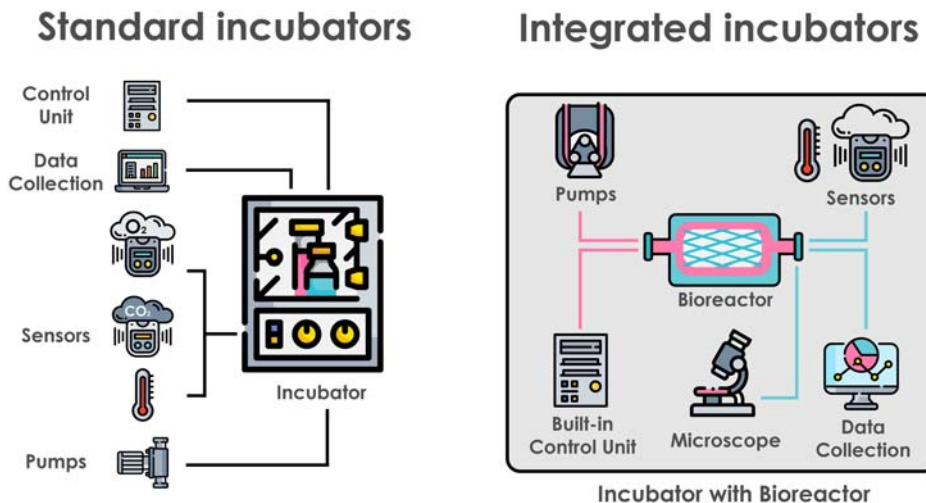


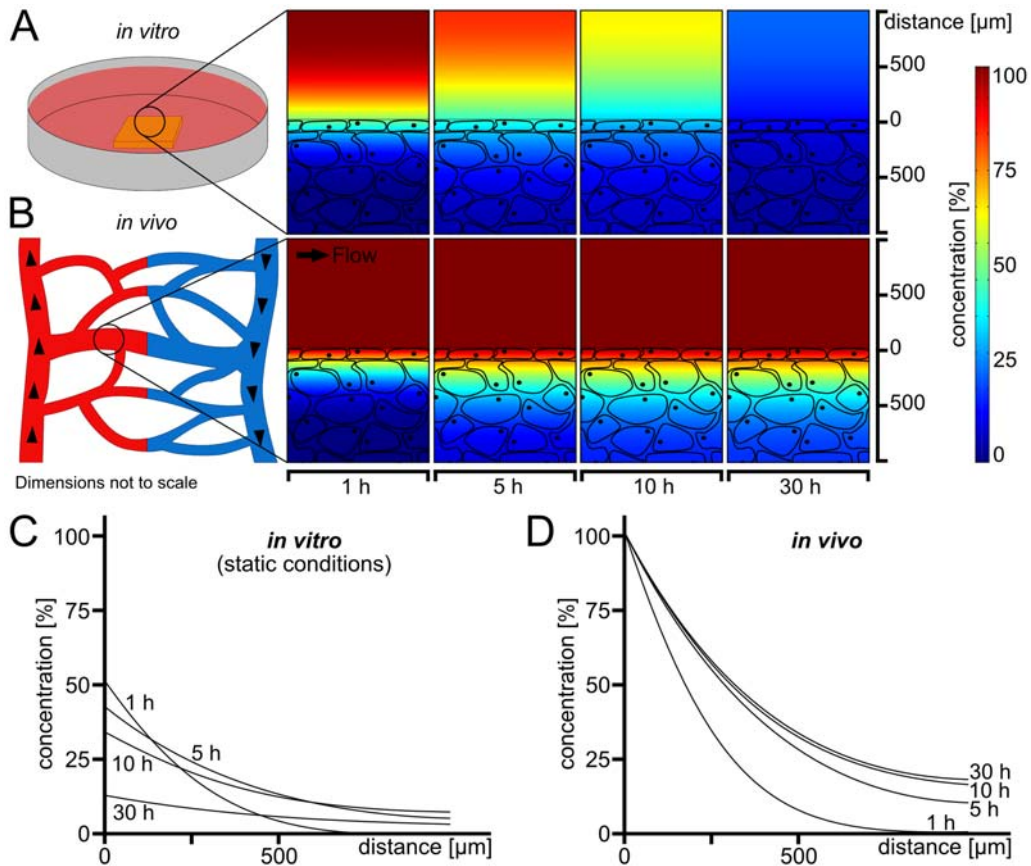
FIGURE 13.2
Traditional standard incubators versus advanced integrated incubator systems.

In addition, bioreactors should be equipped with sensors to enable monitoring and control of crucial culture parameters. These parameters can vary, depending on the culture process. However, temperature, atmospheric CO₂, dissolved O₂ and pH must be monitored and controlled in almost all bioprocesses. Biomass, pressure, impedance, glucose, and ubiquitous metabolites such as lactate are parameters that can be of further interest in tissue engineering. Optimally, sensors should measure on-line and **noninvasively**. Most of the available sensors measure in the circulating media and not in the 3D structure itself although the target parameter might be highly diluted in the media, compared to the inside of the cell-matrix construct. Therefore, noninvasive on-line monitoring of crucial parameters inside the 3D cell-scaffold is urgently needed but remains challenging. If suitable sensors are available, their data can feed a control system which can regulate and document the bioprocess. This can greatly contribute to the robustness and finally the success of a tissue engineering process.

13.4 Mimicking physiological culture conditions

13.4.1 Mass transport

When a tissue is cultured in vitro, a basic requirement for cell viability and development is sufficient **mass transport**. Mass transport fulfills three functions: First, nutrients are delivered to the cells. Second, waste products are removed. Third, information between cells is exchanged via soluble factors (see Chapter 14). Hereby, diffusion is the primary driving force but also the most limiting factor. For example, for a tissue stored under static conditions submerged with cell culture medium: Here the medium serves as a **source** of nutrients such as glucose and growth factors and constitutes a **sink** for waste products (see Chapter 4). Due to a difference in concentration between the supernatant and the tissue, a flux of various factors is established. Inside the tissue, the magnitude of the flux is governed by the diffusion coefficient D and the concentration gradient of the respective molecule (Eq. 13.1). The direction of this diffusive flux is orientated against the concentration gradient—from higher to lower concentrations. During culture, a time-dependent concentration profile is detected. Considering a nutrient concentration in the tissue, the highest value is found at the interface between supernatant and tissue (Fig. 13.3a). With increasing the distance from the supernatant-tissue interface toward the core of the tissue, the nutrient concentration decays (Fig. 13.3c). The reason for this is consumption by cells and diffusion limitations. To model the temporal and spatial development of the concentration pattern, a combination of Fick's second law of diffusion and a reaction term can be applied (Eq. 13.2). Most importantly, the concentration at the supernatant-tissue interface decreases over time. This results in a lack of nutrients inside the tissue. The duration from the initial inflow of nutrients across the supernatant-tissue interface until its depletion depends on the diffusion coefficient and the half-life/**elimination rate**. Assuming a diffusion coefficient of $1.42 \times 10^{-11} \text{ m}^2/\text{s}$ (in the medium) and $1.1 \times 10^{-11} \text{ m}^2/\text{s}$ (in the matrix), which are typical for proteins, and a consumption rate of $6 \times 10^{-5} \text{ 1/s}$ (half-life of approximately 3 h 15 min), the clearance of a factor can already happen 30 h after medium exchange.¹ Additionally, the forming of a gradient in the supernatant limits the flux of nutrients into the tissue. In vivo, the tissue is supplied by the vasculature that distributes

**FIGURE 13.3**

Concentration gradients of a growth factor within a simple in vitro set-up (static) and in vivo.

nutrients and removes waste products very efficiently. In contrast to the in vitro culture conditions, the blood stream maintains consistent nutrient concentrations at the blood–tissue interface. When assuming constant boundary conditions for a nutrient, a steady concentration profile is established (Fig. 13.3b and d). However, even in vivo, the concentration profile shows a limited range into the tissue. This again depends on the diffusion coefficient and the **consumption rate**. To overcome this limitation, capillaries from the vasculature spread throughout most tissues except cartilage, epidermis, and cornea. Considering waste product removal, the blood serves as sink with low waste product concentrations, thereby enabling full depletion of the tissue from metabolites. Communication via soluble factors is also based on diffusion and elimination; nevertheless, cells are the growth factor releasing source, as well as the growth factor binding sink of the tissue (see Chapters 11 and 14).

In the following, the calculation of mass diffusion and shear stress are listed:

Fick's first law

$$\vec{J} = -D\nabla\varphi \quad (13.1)$$

$\vec{J}$ = Diffusion flux (amount of substance per surface and per time), D = Diffusion coefficient in m^2/s , and φ = Concentration (amount of substance per volume).

$$\frac{\partial\varphi}{\partial t} = D\Delta\varphi - k\varphi \quad (13.2)$$

φ = Concentration (amount of substance per volume), D = Diffusion coefficient in m^2/s , and k = elimination rate (amount of substance per time).

Fick's second law with reaction term

The comparison between static in vitro culture and the in vivo situation reveals basic design principles for proper nutrient supply and waste product removal. In order to improve in vitro culture conditions, the volume of the cell culture medium should be chosen very carefully. Importantly, too low volumes result in rapid depletion of nutrients; too high medium volumes can entail negative effects for cells: For example, released soluble factors are diluted in the supernatant and thereby impairing cellular cross-talk. To ensure high flux into and from the tissue, the boundary conditions at the supernatant-tissue interface should be controlled. This can be achieved by dynamic in vitro culture using **perfusion bioreactor** systems which are described in detail later in this chapter.

According to these design principles, perfusion-bioreactor set-ups are harnessed to optimize nutrient supply. Moreover, strategies to embed tube-like structures into a tissue have been developed. The shape and geometries of a bioreactor system show a highly linked development with vascularization approaches. Tools for proper vascularization could be on one hand the generation of channels or vessels by casting, sacrificial structures, 3D bioprinting, postprocessing, or else. On the other hand, porous scaffolds can be used for full perfusion of the tissue graft. Additionally, prevascularized scaffolds have been used for a long time like decellularized intestine, pancreas, or else. The reseeded of those ex vivo structures is promising but do lack geometrical freedom (see Chapter 14).

13.4.2 Physical stimuli

Besides mass transport, many additional factors affect cell viability and development. (see Chapters 3 and 4). Even though most stimuli have more than one effect on the tissue, they can be roughly categorized into distinct types of stimuli (Fig. 13.4): First of all, cells communicate via cell–cell contacts or through secreted signaling molecules. Moreover, the parameters of the matrix or scaffold like stiffness affect the cell behavior. Cells have the ability to remodel the matrix and/or can produce their own extracellular matrix proteins (see Chapters 5, 7, and 8 for more details on ECM and cell–material interactions). The next category of stimuli is provided by the medium: Physical stimuli like shear stress as well as biochemical stimuli like pH, nutrition, CO_2 , and many more are provided via the culture medium. Last, there are stimuli like temperature, strain, or electrical treatments that are applied system wide.

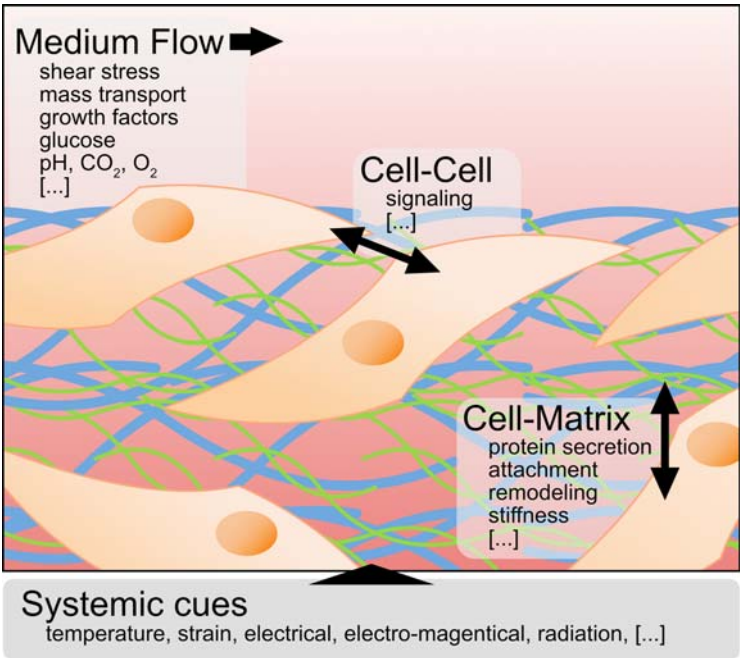


FIGURE 13.4 Selection of physical stimuli with impact to the tissue and tissue maturation within an in vitro bioreactor system.

To suit specific requirements of each type of tissue, these interactions can be tuned to establish *in vivo*-like culture conditions. To guide tissue maturation, most relevant *in vivo* stimuli of the desired tissue have to be identified (Table 13.1). For example, for lung tissue it would be beneficial to simulate breathing, causing a periodic in- and out-flow of air and stretching of the tissue, as well as a liquid–air boundary layer. For vascularization, endothelial cells form a denser blood–tissue barrier when exposed to shear forces and periodic systolic and diastolic pressure. Nevertheless, a bioreactor set-up always renders a simplification of the *in vivo* situation, since an exact mimic is nearly impossible and/or uneconomical.

Even though novel bioreactors aim to be easily adaptable and exhibit universal geometries, current bioreactors tend to be single tissue only.

Table 13.1 Special requirements for different types of tissues.

Tissue	Simplified functionality	Possible stimuli
Lung	Breathing	Air–liquid boundary, stretching, air composition
Muscle	Motion	Stretching contraction, electrical stimulation
Bone	Stability	Strain, weight bearing
Vessels	Mass transport	Flow, pressure, shear stress
Skin	Barrier	Air–liquid boundary

13.5 Bioreactors for cell expansion and cell-based products

Both cell-based therapies and tissue engineered grafts require extremely large numbers of cells. For example, the average cell number per patient and dose of mesenchymal stem cells in current clinical trials is around 100 million cells and usually more than one dose is administered for maximum effect.² It needs an immense amount of time, space, and single-use laboratory plasticware if the cells were expanded in traditional 2D cell culture. Also, harvest of the cells and cell-derived products (i.e., growth factors or extracellular vesicles) is demanding, laborious, or not feasible in 2D cell culture. Hence, improved culture strategies are necessary to allow cost and time efficient expansion of cells in clinically relevant numbers. Several culture systems are commercially available and have been developed to enable specifically the manufacturing of cells. Culture systems that are used in tissue engineering for initiating differentiation and thus maturation of the graft, often by application of mechanical forces, are discussed later in this chapter. However, both types of reactors are part of the tissue engineering process. For example, chondrocytes can be expanded within an expansion bioreactor system *in vitro* to gain the necessary cell numbers for treatment; however, they rapidly dedifferentiate during this expansion. Yet, after the expansion phase, they can be redifferentiated by applying mechanical stimulation onto them via a compression bioreactor. The large-scale production of cell aggregates, spheroids, or organoids in expansion bioreactors is often the first step for many “macro sized” tissue engineering attempts, particularly used as part of bio inks in bioprinting.

Nevertheless, whether it is a tissue engineered, mature graft, cells, or cell-based products, it has to be evaluated in a standardized quality control protocol. Therapies that are already in clinical application are hematopoietic stem cell transplantations as a treatment of hematologic cancers, like natural killer cells, tumor-infiltrating lymphocytes, or modified cells like engineered T cell receptor cells, and chimeric antigen expressing T cells. For these purposes, commercial, good manufacturing practice (GMP) compliant automated or semiautomated bioreactor systems exist: CliniMACS Prodigy, Dyna-Mag, and the G-Rex system. Still in the clinical trial phase are attempts to use stem cells for the treatment of, for example, spinal cord injury, ischemic stroke of the brain, burn wounds, heart infarction, graft versus host disease, diabetes, muscular traumas, and Parkinson’s disease (Table 13.2).

13.5.1 Cell-based products

Recent developments have shown that many beneficial effects of administered cells could also be reproduced by just applying their secreted proteins, growth factors, and extracellular vesicles, which contain among others aforementioned proteins and peptides, but also mRNA. This could omit problems that arise with the transplantation of cells, like immunogenic responses or cancer formation. While cells for therapy require harvesting from the bioreactors, this is not always necessary for production of cellular products. In this context, bioreactors that enable prolonged culture of cells and allow for continuous harvest of the secreted target product are of interest.

13.5.2 Bioreactor types

Different cell types as well as different intended applications of those cells require different culture parameters. Therefore, a multitude of diverse bioreactor systems can be found on the market, with both strengths and weaknesses. The way in which cells can be cultivated is strongly influenced

Table 13.2 Overview on cells for cellular therapies.

Cell type	Cell number	Application	Culture	Phase	References
UCART123 (gene-edited T cells)	Not provided	Targets Cd123 antigen expressed on leukemic cells in acute myeloid leukemia (AML)	Suspension	Clinical phase I	Clinicaltrials.gov, (NCT03190278)
Placental MSCs (PLX-PAD)	$2 \times 10^4 - 1.5 \times 10^8$ cells	Graft versus host disease	Adherent in PLX bioreactor system (perfusion reactor)	Clinical phase I/II	Pluristem.com
Placental MSCs (PLX-PAD)	$1 \times 1.5 \times 10^8$ cells	Muscle regeneration following hip fracture	Adherent in PLX bioreactor system (perfusion reactor)	Clinical phase III	Norgren et al. ³ , Clinicaltrials.gov (NCT03451916)
Tumor-infiltrating lymphocytes (TILs)	4.4×10^{10} cells	Adaptive cell transfer treatment for metastatic melanoma	Suspension in WAVE bioreactor	In vitro	Somerville et al. ⁴
hiPSC-derived cardiomyocytes	7.5×10^9 cells	Bioartificial cardiac tissue formation	Aggregate suspension in DASBox system	In vitro	Jiang et al. ⁵
Adipose MSCs	-	Mass production of hMSCs for therapeutic and industrial applications	Aggregate suspension in stirred tank	In vitro	Egger et al. ⁶
Human skin—derived precursor cells	50×10^6 cells/ 20 cm ² wound	Split thickness skin graft	Aggregate suspension in stirred tank	In vitro	Surrao et al. ⁷
iPSCs	3.6×10^6 cells/mL	Mass production of hPSCs for therapeutic and industrial applications	Aggregate suspension in DASBox system	In vitro	Kropp et al. ⁸
Human embryonic stem cells (hESCs)	3×10^6 cells (mouse model)	Functional pancreatic progenitors for diabetes type I	Adherent in cell factory	In vitro (mouse model)	Schulz et al. ⁹
Human-induced pluripotent stem cells (hPSCs)		Mass production of hPSCs for therapeutic and industrial applications	Aggregate suspension in dynamic mixing (BioLevigator system)	In vitro	Elangew et al. ¹⁰

hiPSCs, human-induced pluripotent stem cells; MSCs, mesenchymal stem cells.

whether they are adherently growing (need to attach to a substrate) or if they can be kept in suspension (for example, blood cells). Microcarriers, spheres with a diameter of 150–300 μm , made from glass, dextran, and synthetic (acrylamide, polystyrene) or biological polymers (alginate, collagen),¹¹ are used to cultivate adherent cells in traditional suspension bioreactors. Next to this, anchorage-dependent cells can also use each other as a substrate, in the form of cell aggregates. Here, the cells are allowed to attach to each other to form multicellular aggregates and produce their own support matrix. Enabling cells to grow in these more natural 3D shapes has gained attention in the last years as a more physiological cultivation strategy.^{12,13} Furthermore, special bioreactor systems have designated surfaces/substrates directly integrated into the system designed to home adherent cells. Some examples of bioreactors are presented in Figs. 13.5 and 13.6, including stirred tank bioreactor, spinner flask, vertical wheel, hollow fiber (HF), and meandering perfusion reactor, and will now be further described in more detail.

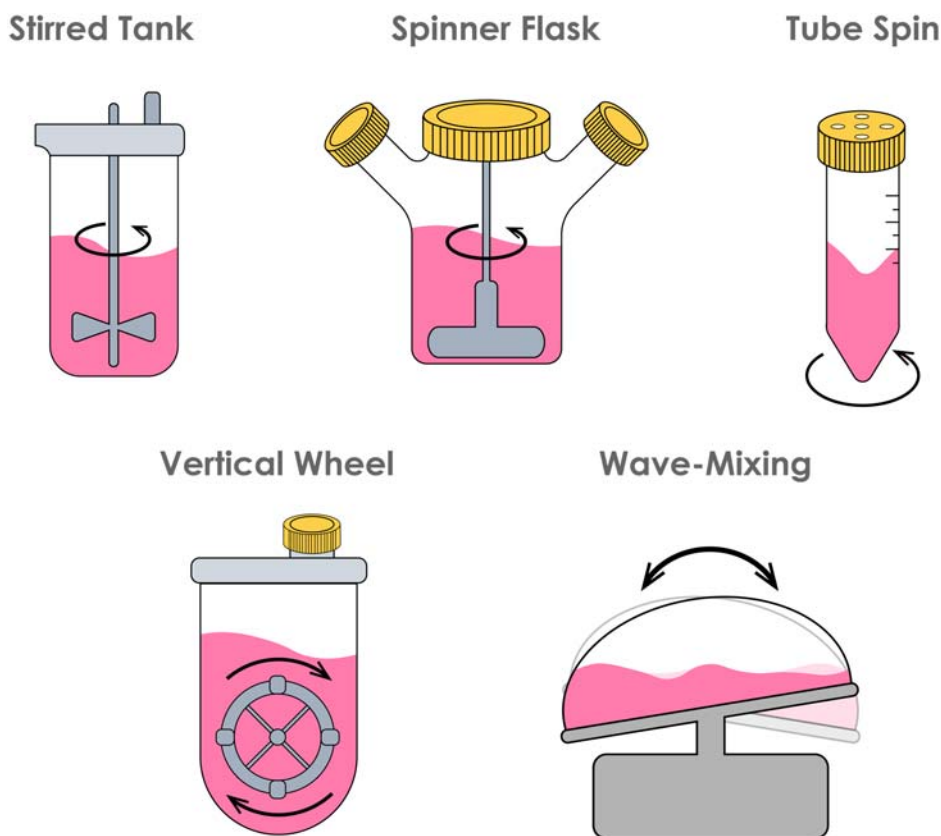


FIGURE 13.5

Mixing bioreactors.

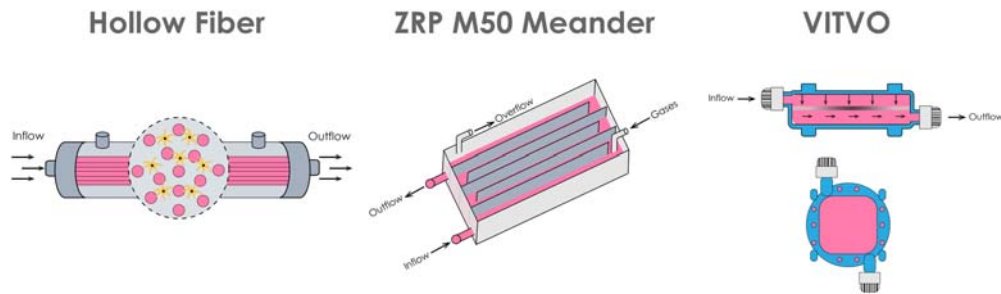


FIGURE 13.6
Perfusion bioreactors.

13.5.2.1 Mixing bioreactors

The most prominent group are **mixing bioreactors**. As their name implies, they homogeneously distribute nutrients, oxygen as well as cellular byproducts via stirring or oscillating and rocking components. Depending on cell type, they can be kept in suspension by mixing or if they need a substrate, grown on microcarriers, which in turn are kept in suspension. Mixing bioreactors can be categorized in three classes. The stirred tank bioreactors (ambr 250 modular—Sartorius AG, Mobius CellReady—Merck Millipore, BioBLU—Eppendorf AG) and spinner flasks (Corning Inc.) have a cylindrical or round bottom shaped vessel, respectively, with a vertical rotor or impeller. Vertical wheel bioreactors (PBS Biotech) are equipped with an upright paddle wheel that are driven by a physical connection, buoyant forces of gas bubbles sparged into the system, or by magnetic force. All three rotor-based mixing reactors are available commercially from multiple suppliers at research as well as industrial scales. The smaller benchtop bioreactors can often be placed in a standard incubator to control temperature and atmosphere, whereas larger bioreactors are equipped with their own, independent control systems. Cell growth is highly dependent on central properties like vessel geometry, aeration, and impeller geometry. Especially, the latter has great influence on mixing time, power input, and generation of shear forces. Therefore, attention must be directed at potential damage or modulation of the cellular differentiation potential. Here, the application of computational fluid dynamic simulation tools can give valuable information on peak forces and help narrow down the optimization range of the stirring speed where proper mixing and separation of the single microcarriers or cells is given but not too high to cause any adverse effects.¹⁴

Wave-mixing bioreactors use a flexible, gas permeable culture bag placed on a rocking platform to mix the liquid inside the bag, and represent the next large class of mixing bioreactors. Compared to most stirred systems, this is a rather gentle way of mixing with usually less shear forces and does provide a bubble-free aeration. Again, different sizes from 1 to 500 L are available.

Another example, that showcases how simple yet efficient a bioreactor system can be, are spin tubes. The components of these systems are disposable, cylindrical cell culture tubes with a conical bottom and ventilated caps that do not need any additional platform but are simply placed on an orbital shaker within a standard incubator. Cells or microcarriers are kept in suspension by the rotational motion of the shaker and can easily be harvested by centrifugation directly in the spin tubes. Different volumes from 10 up to 400 mL per tube are available.

13.5.2.2 Perfusion bioreactors

In comparison to the aforementioned mixing bioreactors, where distribution of oxygen and nutrients is achieved by homogeneous blending, in perfusion bioreactors, a directed flow is applied to the cells or cell-seeded scaffolds. Additionally to the supply with fresh medium, this flow can also translate distinct shear stress to the cells, a mechanical stimulus that is also used to induce differentiation or enhance release of growth factors or extracellular vesicles.

Examples of simple perfusion bioreactors are fixed- or packed-bed bioreactors. They comprise a culture vessel, tightly packed with microcarriers or other scaffolds for adherent cells, that is continuously perfused with fresh medium. With this strategy, a higher productivity can be achieved compared to culture in stirred tank bioreactors due to higher cell densities. However, scale up is more difficult for fixed-bed systems and consequently these systems have an upper limit of 10–30 L. Commercial examples include iCellis (PALL) or Pluristem's proprietary production system. The perfusion rate must be chosen carefully to ensure nutrient supply while retaining low shear forces. Fixed-bed bioreactors are already in use for cell therapies in clinical trials (Table 13.2, i.e., PLX-PAD Pluristem).

HF bioreactors represent another class of perfusion bioreactors. They consist of a bundle of parallel, semipermeable HFs assembled in a cylindrical cartridge. The characteristics of the fiber membrane-like permeability can be tailored to the intended application and is usually given as molecular weight cut off. Thereby, the transfer through the fiber membranes can be tailored to the application. This reactor type also allows for various flow regimes as well as cell seeding possibilities. Offering an intracapillary and extracapillary lumen, cells can be seeded on either or on both sides of the fiber membrane (Fig. 13.6) and allows for coculture of different cell types, where they can receive signals from each other and still retrieve them separately at the end of the culture. The two compartments can additionally be kept static or perfused. In addition, different speeds or patterns on the inside and outside of the capillaries can be established, making this system an extremely adaptable environment.

The ZRP culture system from Zellwerk is a closed system supporting the application of different culture vessels within the same platform, based on perfusion. For example, the horizontal bed reactor is a cylinder with cell carrier sheets on the inside. Cells can grow on both sides of each sheet, while being adequately supplied with medium by gentle circulation of the bed. In the same system, a meander reactor can be installed. This reactor is intended for the culture of nonadherent human cells and tries to mimic the flow in blood vessels. For this, meandering channels in a rectangular vessel are perfused. The vessel is cell repellent, and the main focus of the system is the culture of suspension cells (mainly HSCs), which reside on the vessel bottom while stimulated gently by laminar flow of the medium. The system is GMP compliant and can be used to produce cells for therapies; however, both vessel types are single use plastic and thus have to be replaced after each culture run.

Lastly, the VITVO (Rigenerand) bioreactor is a miniaturized 3D perfusion reactor that creates an in vivo-like environment in a closed system. It consists of a rectangular frame with two transparent oxygenation membranes, allowing gas exchange and visual access during the whole culture. Inside is a fiber-based matrix (thickness: 400 μm), composed of a synthetic polyester separating the culture chamber. Consequently, the two compartments are accessible for cell seeding and medium flow through an inlet and outlet on each side, respectively. Liquid is filling up one chamber first and enters the second chamber

after passing through the matrix. Therefore, cell seeding of the 3D matrix is uniform and easy by applying the cell suspension through the inlet, same for the medium exchange. Furthermore, if cells are prestained with a fluorescence dye, it can be directly viewed under a microscope. The simplicity of design ensures adaptability to common laboratory equipment which benefits academic and industrial research and development fields. This system is however rather designed for drug screening or initial testing runs, as it is not intended to be scaled up. However, due to its ease of monitoring, it can play an important role in developing patient-specific therapies or be used for continuous production and harvest of cell-based products like exosomes.

13.5.3 Scale-up versus scale-out

As already mentioned during the description of the different reactor types, there is great interest in transitioning from research scale to large/industrial scale production. This step is of great importance and highly critical during product development if the respective therapy should ever become a cost-effective, widely used treatment. Hence, it is central that large batches with high cell yields can be manufactured reproducibly. **Scaling up** also reduces the operator-dependent variability and therefore ensures product quality.

However, increasing batch sizes is not the solution for all cell-based therapies. Allogenic therapies use cells from another individual, preferably a young healthy donor, which are administered to a matching patient. In contrast, autologous therapies use the patient's own cells, which are optionally modified before expansion and readministration. This means allogenic cell products can be produced in large quantities and given as an off-the-shelf product to many different patients. However, autologous treatments are limited to one receiver, one lot, and one treatment dose. For these patient-specific treatments, a different strategy is necessary—“**scale-out**.” Rather than larger volumes, parallel smaller manufacturing units are necessary to treat more patients (Fig. 13.7). Examples of such scale-out are the DASBox system by Eppendorf and the Cocoon platform by Lonza.

13.6 Bioreactors for tissue engineering

Now that the initial hurdle of acquiring a relevant cell number is accomplished, especially in tissue engineering, two aspects have to be considered for further graft/treatment production: (1) combining cells with the scaffold (hydrogels, porous materials, etc.) and subsequently (2) differentiation of the cells toward the target tissue.

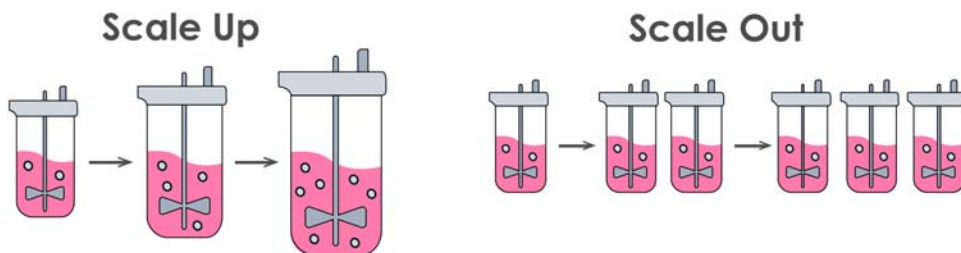


FIGURE 13.7

Scale-up versus scale-out approaches.

13.6.1 Cell seeding

Although it seems trivial, but before a graft can be differentiated first and foremost it has to be assembled. Often the initial cell seeding strategy has a major impact on the outcome of the tissue quality. Larger 3D constructs often have complex geometries, which can make uniform cell seeding challenging. With **dynamic seeding**, cells are introduced to the scaffold by an active motion of the seeding cell suspension. This can be achieved by centrifugal forces or rotation and spinning of the scaffold in the cell suspension (i.e., on a shaker platform). Another method is the utilization of pressure differential for seeding, which can be applied by a bioreactor system. Using a perfusion bioreactor can help to seed (rigid) porous scaffolds more evenly and effectively, also seeding within mixing bioreactors can enhance seeding efficiency. However, depending on the matrix to be seeded, higher cell densities at the surface than within the material were detected when random convection was used.¹⁵ These techniques can greatly improve the penetration of cells into the scaffold and assure a homogeneous distribution, which is essential at the start of a tissue engineering process.

13.6.2 Differentiation

While expansion reactors are required to keep the cells in their initial, native state, bioreactors for tissue engineering are especially built to give the cells cues to induce maturation toward a specific lineage. Research from the last decade proved without any doubt that when biological, chemical, physical, and mechanical cues mimic the physiologic environment, cellular behavior changes dramatically. Mechanical forces in a physiologic relevant range represent important environmental stimuli. Physical forces have a magnitude and a direction and can be distinguished between **shear**, **compression**, **strain**, and **hydrostatic pressure**. A combination of these results in bending or torsion (Fig. 13.8). Mechanical stress is an important factor for guiding cells during differentiation and can result in secretion of extracellular matrix molecules. Spatial organization of cells as seen in muscle and tendon or layered structures such as skin is a direct consequence of mechanical stimulation. In Table 13.3 a short overview of

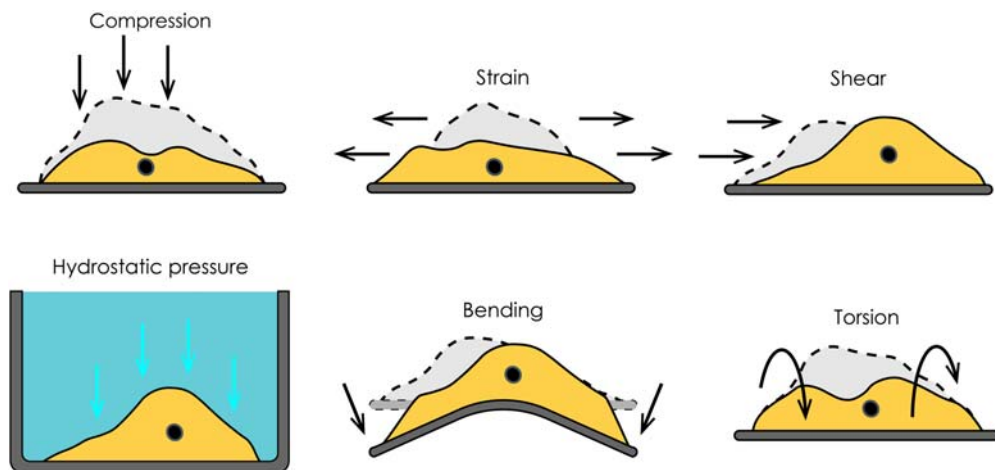


FIGURE 13.8

Mechanical forces acting on cells.

Table 13.3 Characteristics of mechanical forces in the human body.

Mechanical force	Induction of	Physiologic range	References
Compression	Chondrogenic differentiation	10–20 MPa	Hodge et al. ¹⁶
Tension	Osteogenic chondrogenic, ligament differentiation	15–30 MPa	Schechtman et al. ¹⁷
Hydrostatic pressure	Chondrogenic differentiation	5–6 MPa average, up to 18 MPa peak loading	Hodge et al. ¹⁶
Fluid shear stress	Osteogenic differentiation	0.8–3 Pa	Weinbaum et al. ¹⁸

the type of force, the resulting tissue formation, and in what range these forces are found in the physiological environment is presented.

Bioreactors for maturation of tissue engineering grafts are often custom built to fit the exact needs of a research project. However, there is a variety of bioreactors commercially available (Table 13.4 and Figs. 13.9, 13.10 and 13.11). The systems can provide the user with mechanical stimulation in the form of compression for cartilage engineering, tension especially for ligament and muscle engineering, hydrostatic pressure often used in chondrogenesis, and also fluid shear stress which can occur within vessels of the blood and lymphatic system and also used to improve bone formation. An overview of commercially available systems is presented in Table 13.4.

The TC3 by Ebers Medical is a versatile platform system with exchangeable scaffold mounts for different tissue engineering applications (Fig. 13.9a). All the chambers are perfused and the TC3 fits into standard incubator systems. Also, the company offers integrated incubator system with built-in peristaltic pumps. The U-CUP by Celtec Biotek AG is a U-shaped tube with a scaffold mount for porous scaffolds. Via a syringe pump, the medium in the tube can be driven back and forth which perfuses the scaffold

Table 13.4 Commercially available bioreactor systems for directed differentiation.

Mechanical stimulation	Commercially available bioreactor systems		
Compression	CartiGen ^a	FlexCell compression ^b	TC-3 bioreactor with compression or strain chamber ^c
Strain/Tension	FlexCell tension ^b	CartiGen ^a	LigaGen ^a
Hydrostatic pressure	CartiGen HP ^a	TC3 bioreactor with hydrostatic pressure chamber ^c	
Fluid shear stress	InFlow perfusion bioreactor ^d	Streamar fluid shear stress device ^b	OsteoGen ^a U-CUP ^e

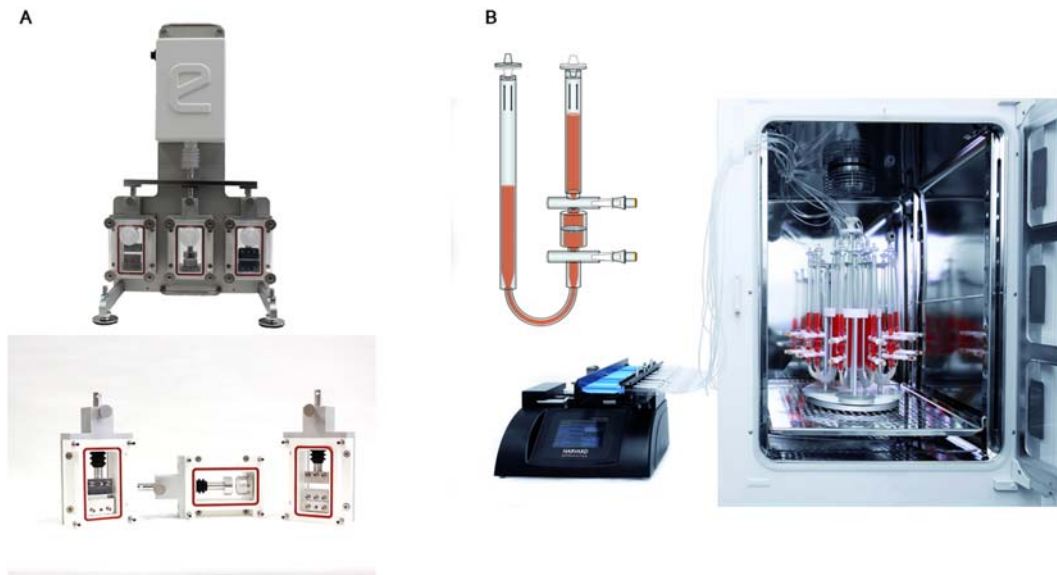
^aBy BISS TGT.

^bBy Flexcell.

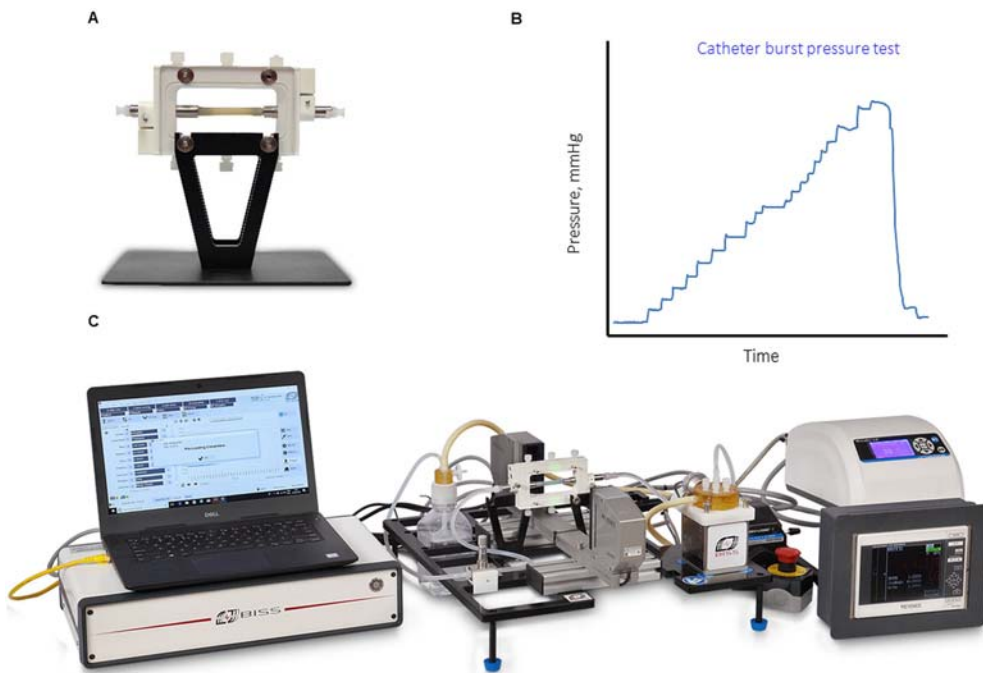
^cBy Ebers Medical.

^dBy SKE Research Equipment.

^eBy Celtec Biotek.

**FIGURE 13.9**

Commercial bioreactor systems. From Cellec Biotech AG.

**FIGURE 13.10**

Commercial bioreactor systems. The LumeGen Pulsatile Stimulation Bioreactor System by ITW India.

**FIGURE 13.11**

Commercial bioreactor systems. The planar bioaxial bioreactor system by ITW India.

and applies fluid shear stress to the cells. Two injection ports (up- and downstream the scaffold) can be used for dynamic cell seeding or sampling. The system can be parallelized so that up to 10 U-CUPs fit into one standard incubator system (Fig. 13.9b).

The company ITW India offers a range of bioreactor systems for the application of compression, strain, fluid shear stress, and hydrostatic pressure. The LumeGen Pulsatile Stimulation allows pulsatile stimulation of vascular tissue grafts, such as arterial grafts at physiological pressure waveforms. The vascular scaffold is mounted in a frame allowing for connection of the graft to a medium circuit (Fig. 13.10a).

Pulsatile stimulation in the LumeGen is achieved through a combination of the precise actuation mechanism and the GrowthWorks application software where the waveforms can be adjusted (Fig. 13.10b). LumeGen bioreactors allow luminal and transmural fluid flow (flow within blood or lymphatic vessels and flow from vessels to the surrounding tissue) to enable mechanobiological studies. These bioreactors have been used for development of arterial grafts and tissue engineered constructs. The LumeGen can be used with a CCD micrometer to measure real time strain as a function of pulsatile stimulation as well (Fig. 13.10c).

Another interesting system is the planar biaxial bioreactor system (also by ITW India). Here, a wide variety of specimens including PDMS sheets, thin films, and tissues can be gripped inside the bioreactor with standard fixtures. This allows for biaxial loading of the scaffold which is especially relevant in cardiovascular tissue engineering and for investigation of tissue viscoelastic properties (Fig. 13.11).

Often, the vessel or scaffold mount of commercially available systems is single use and needs to be replaced after use. On the one hand the use of established systems can save cost and time demanding development processes on the other hand these systems rarely allow for customization and can become expensive over time due to their single-use applicability.

13.7 Future perspective

The field of tissue engineering has experienced substantial advancements and has become a serious discipline with therapies that are already in application. The minimal configuration of a bioreactor comprises the vessel, a scaffold mount, tubing, pumps, sensors, and a suitable incubator system. The entire system should allow to overcome issues of mass transport, ensuring proper nutrient and gas supply through the entire 3D cell-scaffold construct. Further, a proper environment must be established with respect to the physiologic environment of the target tissue. For this, a proper matrix, but also physical stimuli, especially the application of mechanical forces, is required.

Bioreactor set-ups have evolved from large and complicated to smaller and smarter systems with integrated sensor technology that allow for in-process control. Although commercial bioreactor systems for the expansion and differentiation of cells are available, researchers still develop and improve bioreactor systems to enhance the maturation of cells toward a target tissue and to make tissue engineering processes more reproducible. For this, 3D printing in the context of rapid prototyping has become essential to allow for shorter development iterations. Although 3D printing of cell culture vessels and bioreactor parts is already possible, it will need more biocompatible resins in the future. Further, the sensor technologies that are available are mainly focused on measuring parameters in the cell culture supernatant. However, it will need more sensors that measure directly in the cell-scaffold construct, while being noninvasive, to ensure a high quality of the entire tissue. The translation of mechanical forces to the cells is required for a proper tissue maturation. Mathematical modeling can help to estimate these values, but a model is only as good as the underlying assumptions. Thus, sensors that measure the actual forces the cells are experiencing in the scaffold are necessary. This would greatly contribute to ensure the defined application of mechanical forces and thus monitoring and control of the entire process. In the context of industrial and medical applications, more bioreactor systems for the scale-out of tissue engineering processes must be developed to fulfill the future demand of tissue engineered constructs. For this, small simple bioreactor units and a high degree of process automation are required to ensure high-quality products. Again, the key is process control which can only be assured by more advanced sensor technology.

Summary

- Bioreactors for tissue engineering are thought to represent a simplification of the in vivo conditions of the target tissue.
- The minimal configuration of a tissue engineering bioreactor comprises a vessel, a scaffold mount, mechanical actuators, tubing, pumps, sensors, and an incubator.
- Monitoring and control of crucial culture parameters is essential for the development of a tissue engineering process.
- The size of a viable cell-scaffold construct is limited by mass transport of nutrients into and waste products out of the tissue. Thus, mass transport is the most crucial functionality of a bioreactor.
- The bioreactor requirements need to be evaluated for each target tissue separately. There is not "one-fits-it-all" solution to tissue engineering bioreactors.
- Besides the directed differentiation, the manufacturing of a relevant number of cells is an integral part of a tissue engineering process.
- Mixing bioreactors (stirred tank, spinner flasks, vertical wheel, wave) and perfusion bioreactors are the most important bioreactors for the manufacturing of large cell numbers.
- Dynamic cell seeding can greatly improve distribution of cells throughout the scaffold, which is essential at the start of a tissue engineering process.
- Mechanical forces (compression, tension hydrostatic pressure, fluid shear stress) are important stimuli to induce the differentiation of cells into the target tissue. Thus, bioreactors for the directed differentiation of cells are equipped with mechanical actuators to translate mechanical forces.

Classical experiment

Although the cornea is the most transplanted tissue worldwide, availability and quality of grafts are limited due to current methods of corneal storage. A dynamic bioreactor system enables the control of intraocular pressure and culture by air–liquid interface, mimicking the in vivo situation. In addition to a higher degree of tissue viability, healing of epithelial defects was achieved in this bioreactor. First, explanted cornea is fixed in the bioreactor system (Fig. 13.12a) consisting of a main body part that holds the tissue. A fixator keeps the cornea at its position. A lid with septum seals the bioreactor chamber. The septum is connected to one of the two separate medium circuits (Fig. 13.12b). Circuit 1 provides medium to the endothelial cells at the lower side of the

cornea. Circuit 2 delivers medium to the epithelial side of the cornea to mimic tear fluid. The medium of this circuit is collected to a second chamber (bin) below the cornea tissue. Both circuits contain different mediums tailored to the individual cell types. Thereby, the cornea tissue is cultured for up to 7 days without a detrimental effect on the cornea.¹⁹ In the future, additional sensors such as webcams, temperature sensors, pressure sensors, biochemical sensors like pH indicators, and many more can even increase the robustness of the culture process. To manage the acquired data, these sensor data can be analyzed by modern methods such as artificial neural networks to support smart control and error management.

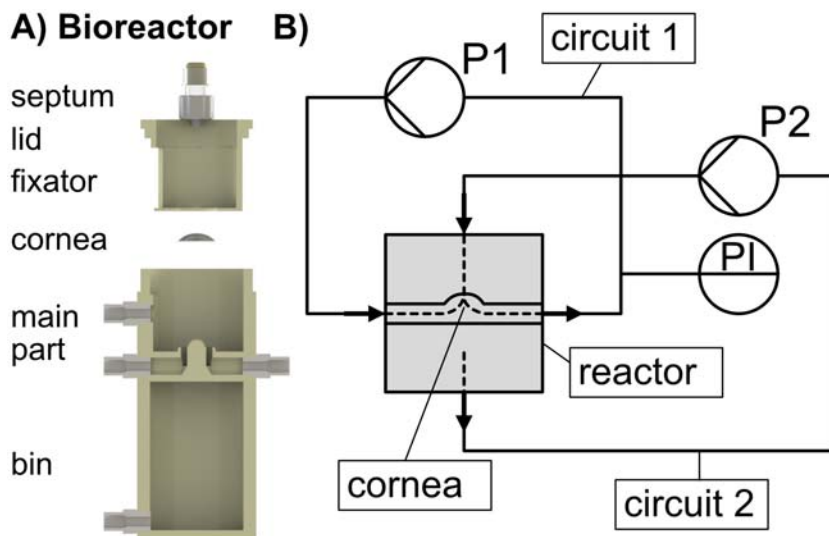


FIGURE 13.12

Bioreactor set-up for dynamic culture of native cornea tissue. From Hansmann et al., 2017. Schmid, R., et al. *In Vivo-like culture conditions in a bioreactor facilitate improved tissue quality in corneal storage*. *Biotechnol J* 2018;13(1):1700344.

State-of-the-art experiment

In this state-of-the-art study, a miniaturized bioreactor for bone tissue engineering was developed and operated in a tailor-made integrated incubator system (Fig. 13.13a).²⁰ In the single reactor set-up, the bioreactor can be equipped with two pressure sensors (P) and a module for the application of hydrostatic pressure (HP; Fig. 13.13b). In the multi-reactor set-up, the parallel culture of up to eight bioreactors in one incubator is possible (Fig. 13.13c). With this system, the authors addressed the need for screening multiple culture conditions to optimize tissue engineering processes.

Human mesenchymal stem cells (MSCs) were seeded on a decellularized bone matrix and cultivated under static and dynamic conditions in osteogenic differentiation medium (ODM) and standard culture medium (CM). The dynamic conditions refer to the culture of a miniaturized perfusion bioreactor at a flow rate that induces fluid shear forces in a physiologic range to the cells. The shear forces were measured and controlled

via noninvasive monitoring of the pressure differential over the scaffold. Interestingly, matrix mineralization, a marker of osteogenic differentiation, was observed in dynamic conditions only. Also, the mineralization in the control medium under dynamic conditions was superior than under static conditions with osteogenic induction medium (Fig. 13.13d). Further, thanks to the miniaturized and parallelizable set-up, several fluid shear stress regimes were screened and optimized. Also, the effect of hydrostatic pressure in combination with fluid shear forces on the osteogenic differentiation was assessed (Fig. 13.13e). For this, a hydrostatic pressure module was introduced to the incubator system and several pressure regimes were evaluated. Although the hydrostatic pressure did not further enhance the osteogenic differentiation of MSCs, the system proved its beneficial attributes for the screening and optimization of tissue engineering bioprocesses.

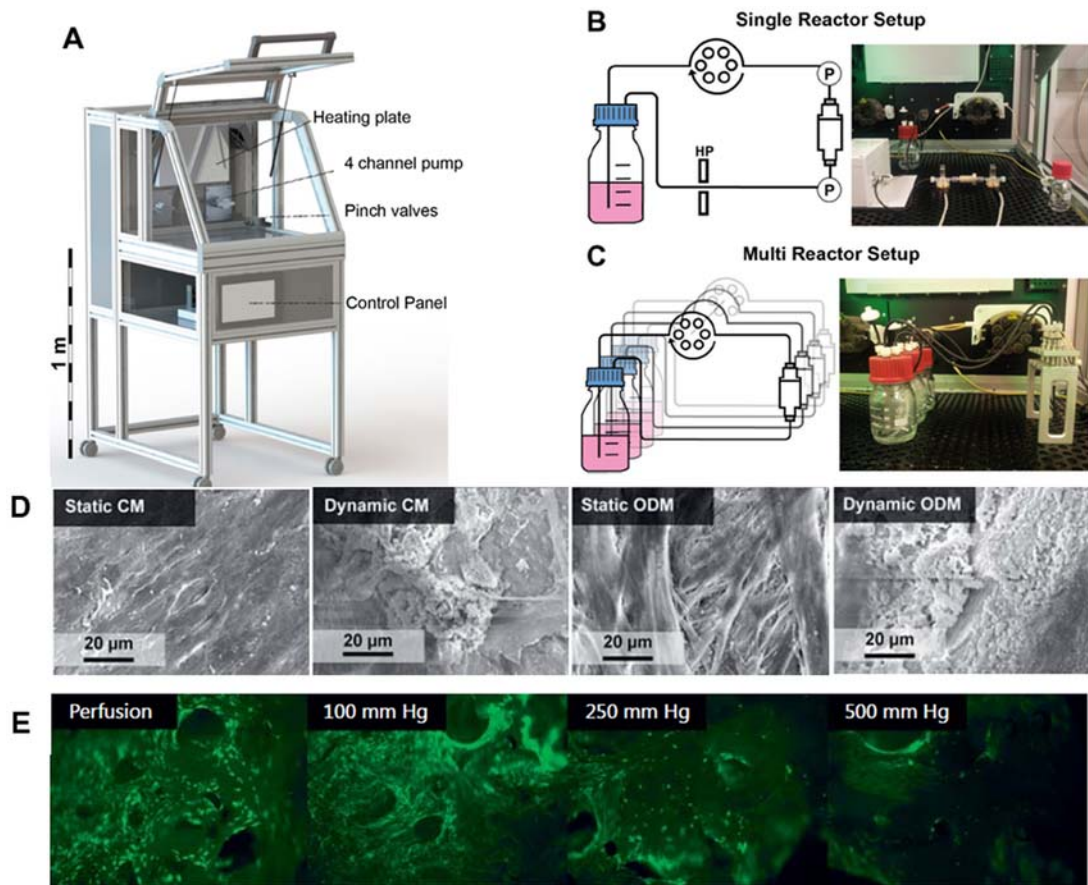


FIGURE 13.13

Integrated incubator with parallelizable miniaturized perfusion bioreactor system for screening and optimization of Tissue Engineering processes. From Egger, D., et al. *Application of a parallelizable perfusion bioreactor for physiologic 3D cell culture*. Cells Tissues Organs 2017;203(5):316–326.

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13.9 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
1. What compounds are necessary to set up a bioreactor system?
 2. What do you need to consider when choosing the material for the tubing?
 3. What are crucial properties of a sensor?
 4. What are the two main classes of bioreactors for expansion of cells?
 5. Why are scale-out strategies more relevant for tissue engineering applications rather than scale-up of a bioreactor?
 6. How can you improve the cell distribution at the start of a tissue engineering process?
 7. Which are the basic mechanical forces applied in tissue engineering?
 8. Which forces are applied for the maturation of which kind of target tissue?
 9. Is copper a suitable material for bioreactors?
 10. How does perfusion increase the nutrient concentration within the tissue?
 11. Which stimuli can be applied via the medium?
 12. How is media perfusion applied in perfusion bioreactor systems and what other bioreactor systems are there establishing motion?

13. Is there a limit for tissue size and if so, what are the limiting factors and how could they be overcome?
 14. Strain, weight bearing, and air–liquid boundary are the most critical stimuli for bone tissue engineering?
 15. Argue whether commercially available bioreactor system are always superior to custom made systems.
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:
1. Describe a general tissue engineering process for the production of a bone substitute.
 2. Why is it challenging to increase the size of a cell-scaffold construct?
 3. Which stimuli could be important for generation of cornea tissue and how would a bioreactor look like?
 4. How would you realize a microscopic real time observation of the tissue inside a bioreactor?
 5. What are the main differences between bioreactors for bioprocess engineering and tissue engineering?
 6. Describe physiological cell culture conditions.
 7. Design a bioreactor system for a tissue engineered bladder and describe the requirements.
 8. Explain Flick's laws and their application in tissue engineering.

Challenge-based learning

Continuous bioreactor to address graft versus host disease

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Human mesenchymal stem cells (hMSCs) have been a promising therapeutic cell source in tissue engineering over the past 15 years. However, hMSC engineering has proven to be more technically complicated than just extracting them, expanding them in vitro, and implanting them back into the patient. This is the reason why many therapeutic strategies with hMSC fail in clinical trials. Extensive research and preclinical/clinical validation are still necessary to make hMSCs a versatile, safe, and effective therapeutic tool. Human *allogenic* mesenchymal stem cells (haMSCs) have been engineered and cultured in bioreactors under highly controlled conditions to study their inherent immunosuppressive capacity. Results have shown that they can be promising therapeutic candidates in graft versus host disease (GvHD). This challenge's vision

is to develop a stable culturing platform to generate readily available and effective immortalized MSCs for GvHD disease treatment.

Motivation and stakeholders

Human stem cells have the potential to become a tissue-engineered therapeutic tool in the future, but precise culture settings need to be established for each medical condition. To generate large numbers of cells, prototype bioreactor systems have been engineered in which cells are seeded onto polymeric particles, which are the free-floating scaffolds on which cells can proliferate. When new particles are added to the bioreactor, cells should move from a full particle to an empty one. This system can ensure continuous cell growth as long as the system is continuously fed with empty particles and the culture conditions are closely monitored. In standard 2D cell culture, confluency is manually monitored by direct viewing of a flask under a light microscope. At the moment, there is no automatic way to monitor the cell occupancy of particles in bioreactors to ensure nonstop growth of haMSCs. The design of bioreactors to grow hMSCs should consider the needs, requirements, and regulatory, financial, and

Continued

Challenge-based learning—continued

technical boundary conditions defined by stakeholders such as patients suffering from GvHD, medical doctors delivering cell-based therapies, stem cell biologists, and bioengineers who manufacture cell culture platforms.

Problem definition

In order to culture human allogeneic MSC, nutrient level needs to be monitored in bioreactors. A proper model and design of a bioreactor to control haMSC's growth and metabolism would need to control the following variables: (a) proliferation rate, (b) nutrient availability and consumption rate, and (c) the rate at which new polymeric particles must be added to the bioreactor.

Challenge

To design a bioreactor system to produce a continuous source of immortalized human allogeneic mesenchymal stem cells as a therapeutic option to mitigate graft versus host disease. Pay close attention in the design of an automatic method to determine the particle occupancy percentage to ensure continuous cell growth.

Learning framework

Reading the Bioreactor chapter and related literature will help you to understand the following:

1. GvHD and the therapeutic potential of haMSCs to treat this medical condition.
2. The different types of bioreactors.
3. Methods to analyze bioreactor variables using sensors and probes.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

4. The parameters that need to be controlled in the bioreactor.
5. Existing biosensors to detect the cell's metabolic shift inside a bioreactor.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

13.10 Glossary

Biocompatible (or biocompatibility) is the ability of a material to perform its function with an appropriate host response without causing any undesirable local and systemic effects.

Bioprocess is a specific process that uses complete living cells or their components (e.g., bacteria, enzymes, chloroplasts) to obtain desired products.

Closed system is a physical system that does not allow transfer of matter in or out of the system.

Compression is the application of balanced inward forces to different points on a material or structure, to reduce its size in one or more directions.

Consumption rate is the average quantity of an item consumed or expended during a given time interval.

Culture parameters are a group of measurable factors that define a cell culture system or set the conditions of their operation.

Dynamic seeding uses agitation or perfusion of the cell suspension to actively increase cell seeding efficiency, uniformity, and/or penetration of cells into the scaffold.

Elimination rate is the rate at which a molecule is removed from a biological system.

Hydrostatic pressure is the pressure exerted by a fluid at equilibrium at a given point within the fluid.

Mass transport (in cell culture) is when materials are moved through the culture medium to the cell surface to deliver nutrients, remove waste, or trigger communication between cells through soluble factors.

Mixing bioreactors are a specific type of bioreactors that homogeneously distribute nutrients, oxygen, as well as cellular byproducts via stirring or oscillating and rocking components.

Noninvasive(ly) is not involving introduction of instruments in the culturing system.

Perfusion bioreactor is a continuous culturing method in which a directed flow of medium is applied to the cells or cell-seeded scaffolds.

Scale-out is adding more components for capacity expansion.

Scaling up is the development of culture systems in stages from (small scale) laboratory to (large scale) industry.

Shear (force) is the component of stress parallel to the material cross-section.

Sink is a reservoir that provides storage for a substance.

Strain is the opposite of compression. Thus, it represents a pulling force applied axially on an object which results in an increase of size in one direction.

Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Strategies to promote vascularization, survival, and functionality of engineered tissues

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14.1 Learning objectives

After reading this chapter you will:

- Develop an understanding of the problems encountered after implantation of engineered tissues.
- Be able to explain the mechanisms of vasculogenesis and angiogenesis.
- Have an overview of the methods currently used in clinics, their advantages, and limits.
- Have an overview of different strategies being investigated to promote fast neo-vascularization of engineered tissues.
- Gain enough appreciation of the problem of vasculogenesis to develop new ideas.

Blood vessels arose during evolution to carry oxygen to distant organs. Not surprisingly, these vessels are crucial for organ growth in the embryo and repair of wounded tissue in the adult.

P. Carmeliet, 2005.

To include an organized vascular network in an engineered tissue, it is important to understand the process of vascular formation and remodeling. A starting point for this process is to look at the formation of the vascular network during embryonic development and growth.

J. Rouwkema and A. Khademhosseini, 2016.

14.2 Introduction

Tissue engineering (TE) is a fast-growing field of research driven by the need for tissue substitutes and transplantable organs to restore their initial function after injury, but also by the need for in vitro models for a better understanding of the target tissues. TE involves both engineering technologies and life sciences toward the development of biological substitutes, which are able to mimic or restore the native target tissue or organ function. Classical TE approaches are based on the association of tissue-

specific cells or stem cells with a biomaterial scaffold, adapted to the tissue to be replaced. Though, even if tremendous progress has been made in material sciences, cell biology, and their interactions, so far, the success of TE constructs is limited to avascular (cartilage, cornea) or thin tissues (bladder, skin), exhibiting a slow metabolism, and mainly relying on passive oxygen diffusion into the tissue.

Clinically, the demand for engineered tissues with controlled dimensions is massive. However, in engineering more complex or larger volumes of tissues, a key challenge is still the inability to provide sufficient blood supply, particularly at the initial phase following implantation, which is a prerequisite for cell viability and long-term survival and functionality of the construct. Upon implantation and until a proper vasculature is established, a tissue graft can only rely on passive diffusion to assure the supply of oxygen and nutrients as well as the removal of metabolites. However, this process is limited to about 150–200 μm of distance to the next supplier vessel. Consequently, a higher cell death rate is often observed in the core of the implant as compared to the outer layer, which is closer to the host vasculature. Such a situation might evolve to improper engraftment and eventually death of the implant. Thus, the long-term survival of cell-based tissue grafts strongly depends on rapid **neo-vascularization**, which is a major hurdle for clinical translation and among the hot topics in tissue regeneration research.

In the human body, the cardiovascular system relies on a hierarchical network of vessels for the distribution of blood pre- and postoxygation (Fig. 14.1). Carrying the oxygenated blood from the heart, arteries branch into smaller arterioles consisting of endothelium leaned on the basement membrane (a thin layer of extracellular matrix proteins) surrounded by smooth muscle cells (tunica media). The blood is further distributed to the capillaries comprising a single layer of **endothelial cells** surrounded by a thin basement membrane that could be either continuous or **fenestrated**. Such thin walls allow for the exchange of oxygen, nutrients, and waste products with tissues. Capillaries then lead back to post-capillary venules that converge into larger collecting veins and finally transport the blood back to the heart. The development of such a system mainly takes place during embryogenesis and only to a limited extent in postnatal life (see Chapter 3 Tissue formation during embryogenesis). Neo-vascularization occurs through two different processes: (1) the formation of new vessels from pre-existing blood vessels, referred to as

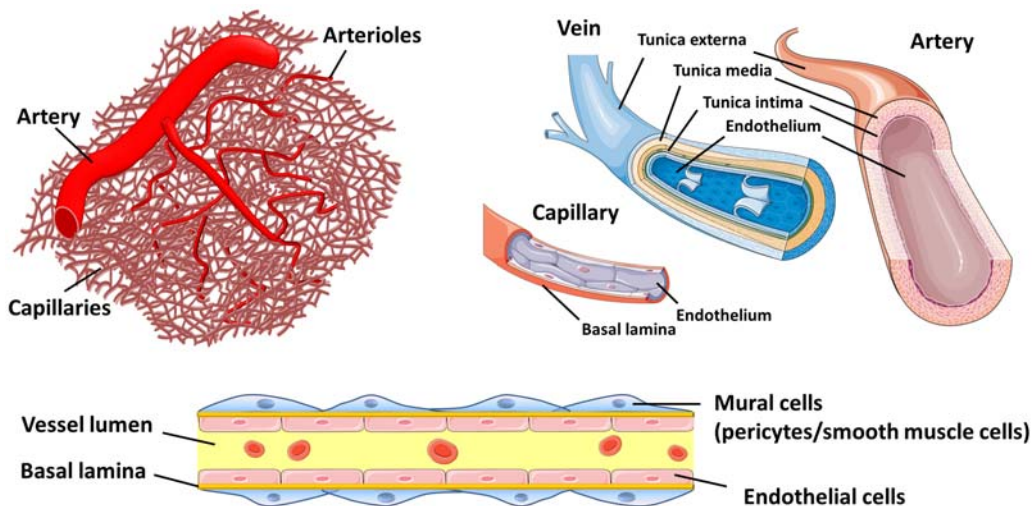


FIGURE 14.1

The vascular tree and the different components of an artery, a vein, and a capillary.

angiogenesis or (2) the de novo formation of blood vessels from **endothelial progenitor cells (EPCs)**, referred to as **vasculogenesis**.

Until recently, vasculogenesis has been considered to be restricted to embryonic development (see [Chapter 3](#) Tissue formation during embryogenesis). However, with the detection of EPCs in adult circulation, it became evident that these cells can also home to sites of neo-vascularization and contribute to vascular regeneration.

In postnatal life, the main neo-vascularization process is angiogenesis. Sprouting angiogenesis was the first identified form of blood vessel formation and occurs in several well-orchestrated steps ([Fig. 14.2](#)).

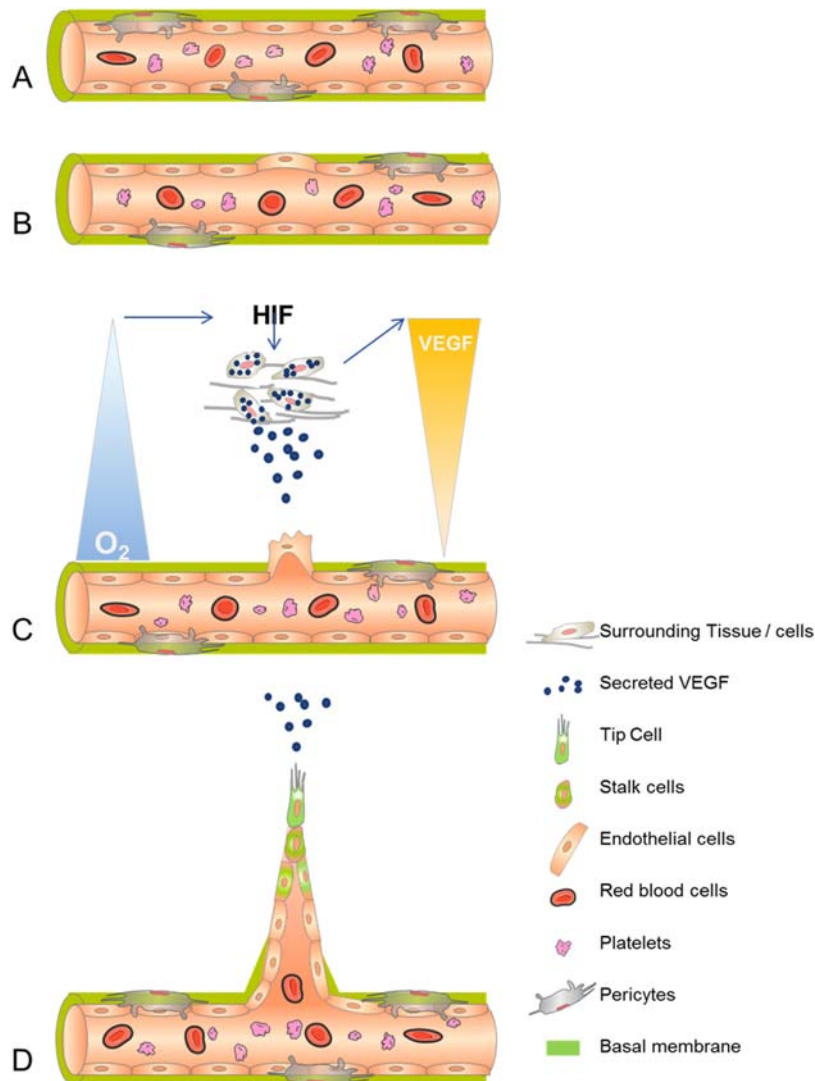


FIGURE 14.2

Schematic illustration of the different steps during sprouting angiogenesis. HIF = hypoxia-inducible factor, VEGF = vascular endothelial growth factor.

Table 14.1 Important factors in angiogenesis.

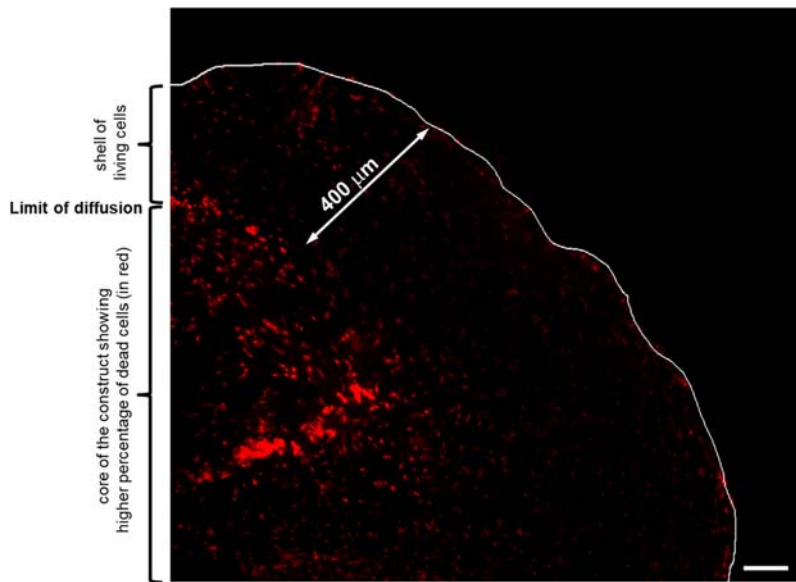
Key factors in angiogenesis	Function
VEGF (isoform 1)	Endothelial cell proliferation, migration, and differentiation
Vascular endothelial growth factor	
HIF	Stimulation of transcription factor of VEGF, PDGFR, TGF- α
Hypoxia-inducible factor (α and β)	EGF, and erythropoietin
PDGF	VEGF upregulation, recruitment and proliferation of pericytes and smooth muscle cells
Platelet-derived growth factor	
Ang-1 Ang-2	Regulation of endothelial cell survival, sprouting and branching of vessels, pericyte recruitment
Angiopoietin	
TGF- α	Induction of angiogenesis and cell proliferation
Transforming growth factor α	
TGF- β	Upregulation of angiogenic factors and proteinases, promotion of basement membrane formation
Transforming growth factor β	
EGF	Upregulation of VEGF production
Epithelial growth factor	
MMP-1, -2 and -9	Basal lamina degradation, extracellular matrix remodeling
Matrix metalloproteinase	
IL-1, -6, -8, and -13	Endothelial cell proliferation
Interleukins	

First, biological signals, known as proangiogenic growth factors (GFs) (Table 14.1), activate receptors expressed on endothelial cells residing in the pre-existing vessels (Fig. 14.2a). Second, the activated endothelial cells start releasing enzymes called proteases, which degrade the basement membrane and allow them to migrate out of the vessel of origin towards the surrounding matrix (Fig. 14.2b). Here, the cells proliferate and form a new luminal structure. These newly developing vessel sprouts then progressively grow and connect to each other to form new blood-perfused microvascular networks (Fig. 14.2c). Third, the newly formed structures are stabilized by the formation of extracellular matrix and the recruitment of cells, such as pericytes and/or smooth muscle cells (Fig. 14.2d).

New vessel growth through sprouting is a slow process with an estimated velocity of about 5 $\mu\text{m}/\text{h}$. This process is sufficient to enable the growth of new vessels across small gaps in the vasculature, but is still not sufficient to elicit vascularization in large 3D TE grafts.

In constructs exceeding 400 μm in any dimension, the lack of rapid vascularization will lead to cell death at the core of the construct and therefore to failure of the implant engraftment within the host tissue, often resulting in fibrous encapsulation and increased scar formation (Fig. 14.3).

Noteworthy, the organization of the vascular network and its maturation are more important than the density of vessels to determine the functional outcome and success of an implanted graft. To create efficient vascular networks, various approaches have been proposed, exploiting cell stimulation via chemical inputs (like GFs) or principles of mechanobiology (like control of scaffold topography/stiffness) and the replication of the natural organization of endothelium through patterning of channels and vascular cells.

**FIGURE 14.3**

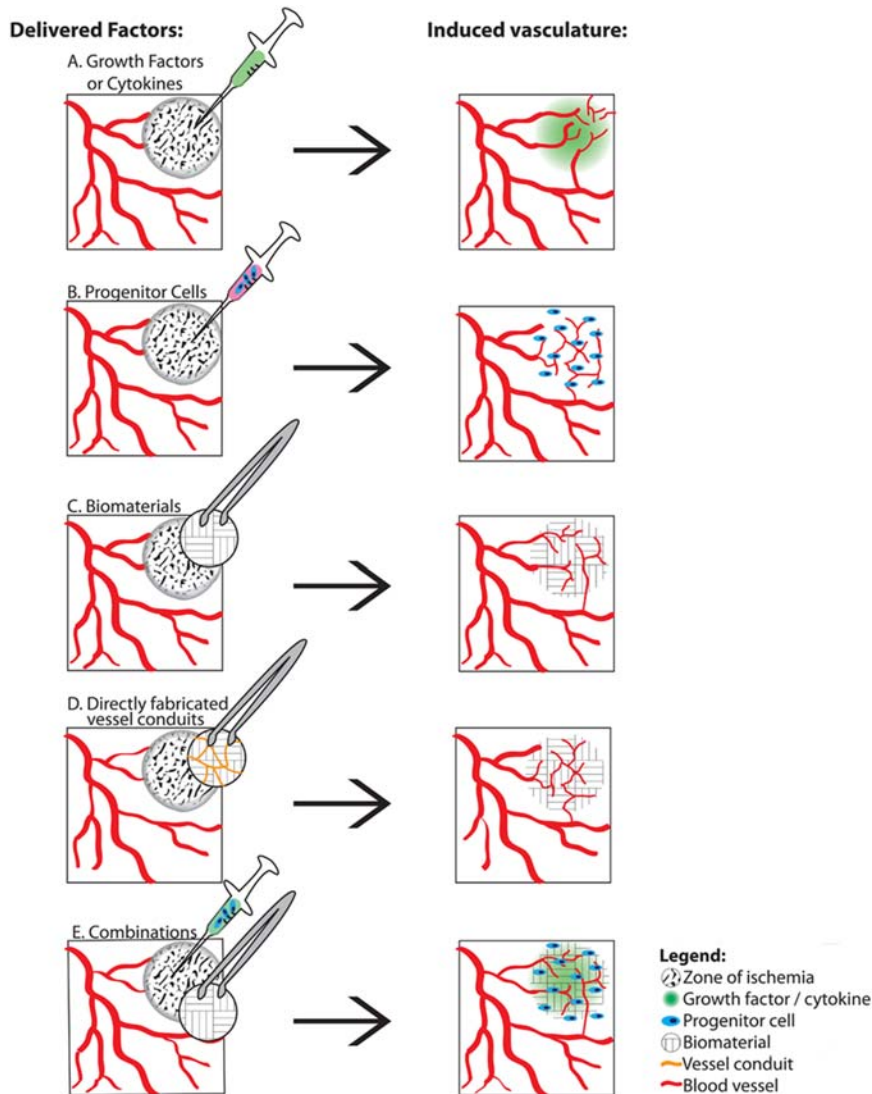
Cell survival is impaired in the core of 3D TE constructs. A disc-shaped hydrogel (diameter 9 mm, height 3 mm) seeded with MSC was cultured for 7 days *in vitro*. Cell viability was assessed on cryosections using ethidium heterodimer staining (red fluorescence) which is only taken up by dead cells with a perforated cell membrane. Scale bar = 100 μm .

In this chapter, we provide an overview of current strategies to improve the host tissue integration of TE constructs with a particular emphasis on the promotion of an early and rapid neo-vascularization (Fig. 14.4). In the first part, we present several *in vitro* procedures to stimulate vascular ingrowth from surrounding tissues; in the second part, we focus on methods to produce prevascularized constructs. After discussing strategies to increase cell viability in the constructs, we describe a few techniques to accurately study neo-vascularization *in vivo*. Finally, major limitations of the current approaches and future directions are discussed.

14.3 Strategies to improve vascular ingrowth into TE constructs

14.3.1 Modification of scaffolds

Scaffolds represent structural units for the generation of TE constructs (see Chapter 11 Scaffold design and fabrication). They serve as 3D matrices for both cellular attachment and proliferation as well as for the development of a functional tissue. The choice of a scaffold that adequately promotes vascular ingrowth is a major step toward the successful integration of the implant. Several properties of scaffolds, such as chemical composition, degradation rate, architecture, porosity, and surface topography, are known to influence the process of vascularization. The progress achieved in biomaterial sciences now enables the fabrication of structures with precisely defined features, which may be used to promote vascularization in the engineered tissues (see Chapter 8 Cell-materials interactions).

**FIGURE 14.4**

Current options to induce vascularization in TE constructs. From Phelps, Garcia. *Engineering more than a cell: vascularization strategies in tissue engineering*. Curr Opin Biotechnol. 2010;21(5):704–709.

Chemical composition

Scaffolds only varying in their chemical composition or degradation rates can induce different reactions in the recipient's body, closely correlating with vascular ingrowth. Surface chemistry, charges, and wettability have an influence on protein adsorption and therefore on cell adhesion. Upon implantation, biomaterials rapidly adsorb proteins from the surrounding tissue and blood, resulting in the

attraction of inflammatory cells that release cytokines and angiogenic GFs that promote neo-vascularization. Although mild proinflammatory properties of scaffolds can be beneficial to stimulate vascularization, those should be carefully tailored to not compromise the biocompatibility of the implanted constructs. For example, for bone TE, scaffolds containing β -TCP (tricalcium-phosphate) are often used due to their chemical composition resembling that of native bone, and their natural degradability following the de novo bone formation.

Sophisticated approaches aiming at the development of scaffolds tailored to the target tissue requirements and at the promotion of adequate neo-vascularization within implants have been developed over the past decades. One strategy is the plasma-spray or plasma-gas treatment of synthetic polymers constituting the scaffolds, such as poly-L-lactic acid. In this technique, a high-energy gas flow gets in contact with a material and consequently modifies the physico-chemical characteristics of its surface, facilitating the adsorption of biomolecules or the covalent grafting of active peptides, which then improve cell-surface interactions. Another example is the precoating of the scaffolds with heparin (an anticoagulant) or heparin-mimetic molecules. Heparin provides anchoring points for proangiogenic GFs, promoting an active neo-vascularization (as opposite to passive neo-vascularization that relies on the natural sprouting or elongation-driven angiogenesis from the surrounding vascular network).

Physical properties

Besides the chemistry of scaffolds, their surface topography and overall architecture (porosity, geometry) also have a strong influence on vascular invasion. Likewise, implants presenting rough surfaces were shown to be surrounded by a higher density of new vessels when compared to smooth implants of the same chemistry. Micro- and nanotopography of materials enable better protein adsorption and thus, cellular adhesion and proliferation.

The porosity of a scaffold (evaluated as pore size distribution and interconnectivity) also represents a key factor for neo-vascularization. Scaffolds with large pore sizes (ranging between 250 and 400 μm) and interconnectivity favor the formation of vascular networks with larger blood vessels, deeper penetration, and higher invading vessel density, which is particularly important to guarantee the survival of cells in the center of large 3D tissue constructs.

A functional (micro-) vasculature consists of a three-dimensionally interconnected and branched network of tubular structures. To organize into tubular structures, cells need to proliferate, elongate, and align. To accelerate this rather slow process, research has also focused on the development of constructs presenting preoriented microluminal or hollow channels mimicking the physiological vascular architecture of tissues, which enhances vascular ingrowth from the surrounding host tissue.

Biological properties—decellularized matrices

Decellularized scaffolds, usually of xenogeneic origin, have been proven to promote both angiogenesis and local regeneration of the damaged tissue (see [Chapter 5](#) Extracellular matrix as a bioscaffold for tissue engineering). Combining appropriate chemical, physical, and mechanical properties, decellularized matrices have long been used in the field of bone TE. Today progress in chemical and enzymatic processes enables the decellularization of a variety of tissues such as blood vessels, cardiac valves, intestine,

and bladder. While preserving the integrity and biological activity of proteins, those technologies also protect the native structure of tissue and the pre-existing vascular lumen, facilitating cellular invasion.

14.3.2 Fabrication of predefined hollow channel networks—new technologies

To fabricate constructs comprising hollow channels, different methods can be used, such as electrospinning, 3D printing (and micromolding), microfluidics, and the recently developed sound-wave technology (see [Chapter 11](#) Scaffold design and fabrication).

3D printing

As mentioned before, the vascular network is organized in a vascular tree, comprising larger vessels branching into smaller capillaries ending in a microvascular network that ensures a physiological blood flow throughout the tissues. Latest advances in biomaterial research, in parallel to the fast-growing technology of 3D printing (layer-by-layer printing), enable the production of complex multi-material structures with precisely tailored material chemistry, scaffold shape, and structure. The further development of biological inks (**bioinks**) allows for the encapsulation of cells or aggregates and their deposition at exact locations within 3D environments, making this technology attractive for the fabrication of prevascularized engineered tissues or organs. Bioinks are mainly natural (e.g., collagens or gelatine), synthetic (e.g., PEG, polyethylene glycol) hydrogels, or a mixture of both (e.g., GelMa, Gelatine Methacrylate). Different bioprinting strategies are currently used to generate vessel-like structures. For example, a lumen can be created within hydrogels using 3D-printed molds featured with a required length, diameter, and branching structure. After curing, the hydrogel is removed from the cast leaving an imprint of the required template (**lithography**). Other methods involve the retraction of needles placed into the gel before jellification, leaving a hollow space that can be seeded with angiogenic cells. Even if relatively easy to achieve, this technique only allows for simple designs. Furthermore, the resulting channel diameters are determined by the size of needles used. Another way to produce hollow channels in 3D structures is the use of pulsed laser beams. When in contact with photo-cross-linked hydrogels, the laser beam degrades covalent bounds at desired locations in the gel, creating precise hollow spaces. This enables the “drawing” of more complex 3D tubular and interconnected structures with defined lumens that can be as small as 10 μm in diameter. Likewise, the use of the so-called sacrificial materials in layer-by-layer printing allows to print complex and precise 3D patterns. The sacrificial ink is then washed away, leaving a perfusable luminal network within the scaffold.

Microfluidics

In the quest for alternatives to animal models for the study of cellular behavior, microfluidic platforms have been developed. Typically, microfluidic chambers are comprised of perfusable microchannels of various sizes embedded into hydrogels, which mimic the targeted tissue composition and mechanical properties. Upon perfusion, the fluid flow causes shear stress that not only crucially affects endothelial cell invasion, matrix production, and orientation, but also stimulates cellular sprouting. Nearly 20 years after the first attempts to include cells inside channels and/or within the surrounding matrix within microfluidic chambers, it is now possible to mimic different organs and related pathologies on chips (organ-on-chip) and sophisticated models enabling the study of complex multicellular systems in a perfused biologically and mechanically relevant environment are now available.

Sound wave technology

All the above-mentioned technologies aim to produce microvascular-like structures in scaffolds or gels through perfusion, injection, or printing of endothelial cells in pre-established hollow channels. Another approach based on the capacity of endothelial cells to self-assemble into vascular networks has recently been developed. This emerging technology uses acoustic vibration to induce precise patterning of cells suspended in hydrogels. Variable sound frequencies can produce different 3D cellular patterns that are stabilized upon subsequent hydrogel jellification. Within such a system, the cells are not subjected to the mechanical stresses induced by extrusion needles.

14.4 Strategies to improve vascular ingrowth into TE constructs—biological features

14.4.1 Incorporation of growth factors

Angiogenesis is a highly dynamic multistep process, involving the activation, migration, proliferation, and tube formation of endothelial cells, followed by the recruitment of perivascular cells stabilizing the newly formed microvessels. Each of these steps is induced and tightly regulated by the cellular release of angiogenic GFs. Many of those have been identified and characterized in terms of protein biochemistry, structure, and biological function. Recombinant forms can be synthesized and are commercially available. Accordingly, angiogenic GFs are often incorporated into scaffolds to promote vascular ingrowth into engineered tissues as their local availability can guide the organization of vascular cells. The most used factors (Table 14.2) are vascular endothelial growth factor (VEGF), basic fibroblast growth factor, angiopoietin-2, platelet-derived growth factor (PDGF), and hepatocyte growth factor. The combination of multiple factors enables the temporal control of vascularization by efficiently assisting the different stages of the vascular network assembly, including the stabilization of endothelial cells and the recruitment of mural cells (see Chapter 4 Cellular signaling).

Rather than the availability of the GFs, their distribution into concentration gradients controls the migration of vascular cells. For instance, patterning of VEGF onto scaffolds or within hydrogels drives the elongation and branching of endothelial cells according to predefined spatial geometries. The strategies for incorporation of angiogenic GFs are several, ranging from direct covalent immobilization to precoating of scaffold surface with GFs. The simplest approach consists of dip coating the solid scaffolds into GF-containing solutions: the differential release of multiple factors, dictated by the diverse binding affinity of the proteins to the surface, can be used to simultaneously promote tissue formation and

Table 14.2 Growth factors incorporated in TE constructs to promote vascularization.

Factors to promote angiogenesis	Factors to promote vessel maturation
VEGF	PDGF
PDGF	TGF- β
bFGF	Ang-1
Ang-2	

vascularization. Another common immobilization strategy relies on scaffold modification through heparin, which binds to GFs via specific motifs of negatively charged sulfate groups. This binding limits the diffusion of GFs while preserving their bioactivity and integrity against enzymatic and thermal degradation. Heparinized matrices delivering VEGF and FGF2 have shown enhanced angiogenesis. Alternatively, the pores within porous scaffolds may be temporarily filled with GF-containing gels, which are replaced by newly formed tissue over time.

Rich of many GFs and easily isolated from blood samples, platelet-rich plasma (PRP) represents a good and cost-effective candidate biomaterial. However, being autologous, its composition is not standardized and may vary considerably from donor to donor. Furthermore, GFs can also be supplied via artificial drug delivery systems, such as microspheres or nanoparticles, bearing the advantage that they exhibit clearly defined GF concentrations and release kinetics (see [Chapter 12](#) Controlled release strategies in tissue engineering). Accordingly, it is possible to temporally control GF signaling and thus, to optimally support individual stages of angiogenic processes, including vessel formation, maturation, and remodeling. Importantly, the carrier-mediated delivery of GFs has proven to be also important for the engineering of scaffold-free vascular constructs. For instance, gelatin microspheres efficiently deliver the transforming growth factor beta 1 (TGF β 1) in self-assembled constructs with a high cellular and extracellular matrix density. Finally, other scaffold modifications can stimulate locally the production of proangiogenic factors. In gene-activated matrices (GAMs), the scaffold is enriched with plasmid DNA that boosts protein production by cells directly into the implantation site. GAMs for regulation of angiogenesis have been already reported for the nonviral delivery of the encoding sequences of VEGF and antisense cDNA of thrombospondin.

14.4.2 Incorporation of vascular or proangiogenic cells

Following the idea that endothelial cells can either spontaneously form a vascular network or cover prefabricated channels, their incorporation in TE constructs is commonly used to promote neo-vascularization.

These preformed vascular structures can then readily connect to the host vascular system after implantation. Major considerations for the choice of an endothelial cells source are the cell availability, their expansion potential, and functional properties.

Mature endothelial cells have an intrinsic high angiogenic potential and various tissues have been used for their extraction. For example, human umbilical vein endothelial cells (HUVECs) or human dermal **microvascular endothelial cells** (HDMEC) have been isolated from the umbilical cord and skin, respectively. Mature endothelial cells show limitations concerning their narrow proliferation potential. Also, some commonly employed cell types (*e.g.*, HUVECs) are not compatible with an autologous setting, meaning that caution has to be taken to avoid immunogenicity and rejection. In addition, only a limited amount of material can be harvested from healthy donors. These hurdles may be overcome using **induced pluripotent stem cells** (iPSCs), which are also a promising cell source for cardiovascular TE (see [Chapter 18](#) Principles of cardiovascular tissue engineering). Another promising source of endothelial cells is the EPC population. Involved in the prenatal vascular network formation (see [Chapter 3](#) Tissue formation during embryogenesis), EPCs are also found in postnatal peripheral blood and bone marrow. The use of EPCs may be of advantage for clinical applications as they can be

obtained from autologous sources and display high proliferation capabilities. Two cell populations referred to as early EPCs or myeloid angiogenic cells and late outgrowth endothelial cells (OECs) or endothelial colony forming cells have been isolated from peripheral and cord blood. Early EPCs contribute to neo-vascularization by **paracrine** mechanisms, while OECs are actively integrated in newly formed vessels. Besides blood, the isolation of EPCs from bone marrow and stromal vascular fractions (SVFs) of adipose tissue is also possible through various protocols based on the expression of surface markers, such as CD31, CD34, CD133, and VEGFR2 (CD309). EPCs from different sources are part of clinical trials for the treatment of ischemic diseases with promising early phase results. However, a major hurdle in further developments of successful therapies is the heterogeneity of EPCs, which requires a functional and detailed characterization of the cell population (Fig. 14.5). In addition, the expansion of endothelial cells and EPCs requires specific, recombinant GF-containing cell culture media, which is associated with additional costs.

Functional cell characterization can be assessed by their capacity to form tube-like structures in presence of **Matrigel**, a gelatinous mix of proteins also present in the basal membrane. In standard protocols, endothelial cells are seeded on a thin layer of MatrigelTM, and tube formation is observed over 24h (Fig. 14.5a). From a molecular point of view, important functional characteristics are the uptake of acetylated low-density lipoprotein particles (Fig. 14.5b left panel) and binding of UEA-1 lectin (Fig. 14.5b right panel), which can be observed by fluorescence microscopy or flow cytometry.

Mural cells, such as vascular smooth muscle cells surrounding the arterioles, or pericytes wrapping the capillaries and microvessels, support angiogenic sprouting, stabilize newly formed vessels, and assist vessel maturation (see [Chapter 18 Principles of cardiovascular tissue engineering](#)). TE constructs including a mural cell source have been proven beneficial in terms of stability of the newly formed vascular network. Interestingly, the **interplay** between endothelial cells and perivascular cells, as for progenitor cells and stem cells in bone, has been shown to play an important role in tissue homeostasis. In bone TE, mesenchymal stem cells (MSCs) or primary osteoblasts are commonly used as an osteogenic cell source (see [Chapter 16 Cartilage and bone regeneration](#)). Of interest, the coculture of these 2 cell types facilitates neo-vascularization, where MSCs/osteoblasts act as mural cells.

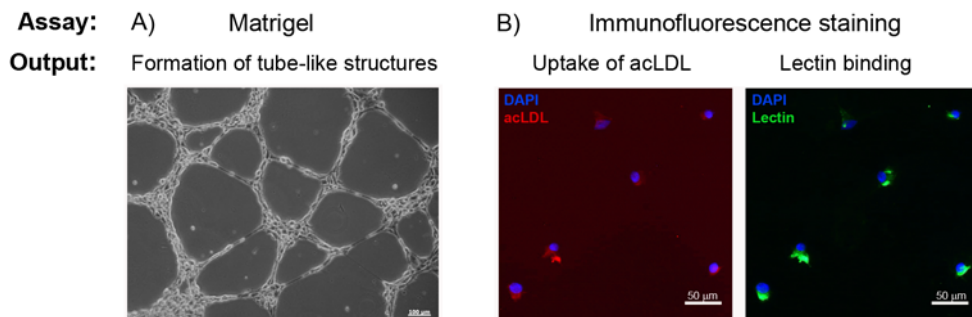


FIGURE 14.5

Functional characterization of endothelial cells.

Finally, another cell-based strategy involves the genetic manipulation of tissue-forming cells to overexpress proangiogenic factors and ensure their long-term release. However, concentrations of angiogenic factors exceeding certain threshold levels have also been shown to be detrimental.

14.4.3 Incorporation of microvascular fragments

Adipose tissue-derived **microvascular fragments** (MVs) have recently been introduced as very potent vascularization units. MVFs represent a randomized mixture of biologically intact arteriolar, capillary, and venular vessel segments that can be isolated from adipose tissue by applying enzymatic digestion (Fig. 14.6). After isolation, MVFs can be seeded onto TE scaffolds before implantation into the desired target tissue. When compared to single cell-based approaches, the MVFs offer several advantages, such as a rapid isolation procedure and reduced exposure time to enzymes (only ~ 10 min), resulting in much higher viability ($\sim 95\%$) when compared to single cells ($\sim 80\%$) extracted from identical tissue. This aspect, in combination with the presence of physiological niches containing resident stem cells, determines a very high proliferation rate and differentiation capacity of cells in MVFs after implantation.

The most compelling argument for the use of MVFs is the maintenance of their physiological structure after isolation, including intact segments of the vasculature that consist of a central lumen, endothelium, and even stabilizing cells. With a length of $20\text{--}150\text{ }\mu\text{m}$, MVFs can bridge wide distances within seeded scaffolds. This is a crucial benefit when compared to the relatively slow growth rate of angiogenic sprouts penetrating the implanted scaffolds from the surrounding host tissue. Following their implantation, MVFs immediately form interconnections with each other as well as with the vasculature of the host tissue, ultimately leading to the development of stable and blood-perfused networks without additional *in vitro* precultivation. Nevertheless, although numerous *in vivo* animal studies have shown the successful use of MVFs as vascularization units, this approach has not been transferred into clinical practice yet.

14.5 Strategies to promote neo-vascularization

14.5.1 In vitro prevascularization

In vitro prevascularization of TE constructs relies on the assembly and prematuration of vascular structures *in vitro* prior to implantation. This approach is of great interest, as **anastomosis** of vascular fragments with the host vascular system occurs much faster than the *de novo* vessel formation. A

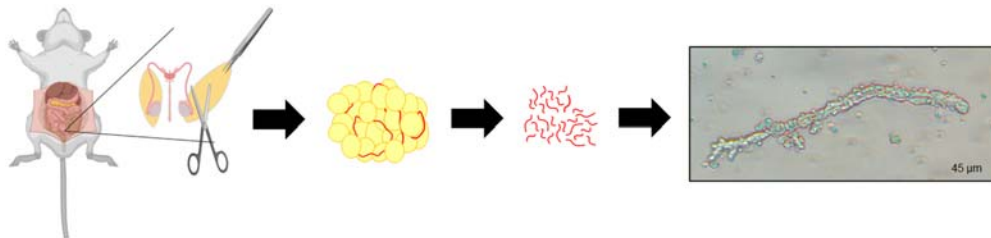


FIGURE 14.6

Microvascular fragment isolation from mice fat pads.

prevascularization step is primordial when endothelial cells or EPCs are chosen as a cellular source, while it may be omitted when MVFs are applied (Fig. 14.7). Upon implantation, the prevascularized constructs only need to develop connections (**inosculations**) with the host microvasculature and therefore achieve faster blood perfusion.

Different strategies have been developed to support the formation of vascularized structures within constructs seeded with endothelial cells or EPCs. This includes the application of (modified) scaffolds as well as the culture of cells in spheroids or cell sheets (Fig. 14.7). Endothelial cells and progenitor cells self-assemble spontaneously into capillary-like structures *in vitro*. However, since the resulting structures are often unstable, the coseeding with other vessel-stabilizing cells has been proposed. Prevascularization of scaffolds has been extensively applied in bone and skin TE. For instance, endothelial and osteogenic cells (such as MSCs) can be coseeded into porous scaffolds to promote bone regeneration.

Prevascularized multicellular **spheroids** can be generated by several techniques (e.g., pellet cultures, spinner cultures, liquid overlay, and microfluidic approaches) where the cells aggregate and undergo self-assembly. Being free of exogenous material, spheroids can serve *in vivo* as paracrine stimulators of angiogenesis or as endogenous building blocks for more complex structures, just like prevascularized microtissues and branched vascular trees in macro tissues. **Cell sheets** are another scaffold-free option for TE constructs. Their fabrication takes advantage of the extracellular matrix produced by cells themselves. Vascularized cell sheets can be generated by sequential assembly of cell layers from endothelial cells and tissue-specific cells (see Chapter 5 Extracellular matrix as a bioscaffold for tissue

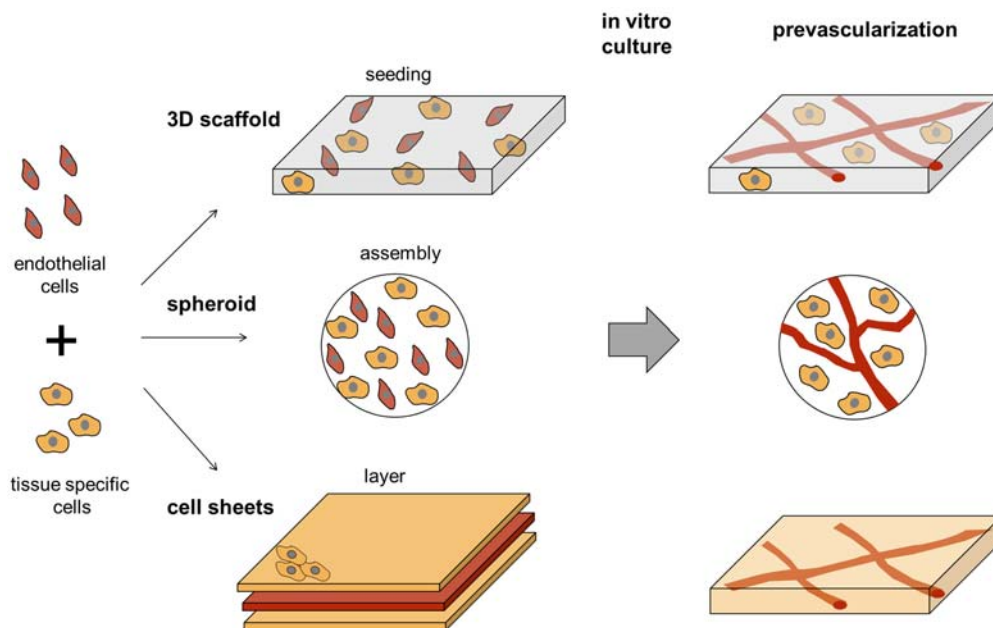


FIGURE 14.7

Schematic representation of common prevascularization strategies.

engineering). Dermal substitutes developed through the coculture of OECs and fibroblasts into 3D sheets have been proven effective in the treatment of patients with large skin defects. Prevascularized mucosal cell sheets promoted cutaneous burn wound healing with accelerated wound closure, more microvessels and proliferating cells, and reduced scarring, fibrosis, and neutrophil infiltration as compared to silastic sheets and skin grafts. The engineering of prevascularized cell sheets has also allowed for the construction of cardiac and bone tissue.

Decellularized tissues have also been used for various tissue repair approaches. Composed of extracellular matrix, these materials can guide the formation of angiogenic vessels upon engraftment (see [Chapter 5 Extracellular matrix as a bioscaffold for tissue engineering](#)). For example, being osteo-inductive and promoting direct osteoblastic differentiation, decellularized native bone matrix is a scaffold of choice in clinical bone TE; seeded with cells it promotes direct bone matrix formation (intramembranous ossification). However, such material fails in large constructs, due to the lack of vascularization. The use of decellularized hypertrophic cartilage matrix has also been explored for its potential translation to orthopedic applications, aiming to mimic endochondral ossification, a process involved in long bone development. Here, the chondrogenic cells differentiate toward **hypertrophy** secreting proangiogenic factors that promote the vascular ingrowth from surrounding tissue, and the bone matrix is eventually deposited. Of note, this neo-vascularization strategy is based on the natural capacity of chondrocytes to survive in poor oxygen and nutrient environment.

14.5.2 Strategies to improve cell survival

Beside the above-described methods to promote and accelerate the neo-vascularization of TE constructs, several strategies have been reported to improve the survival of transplanted cells by **in vitro preconditioning**. On the one hand, these approaches use chemical and biological inhibitors of major cell death pathways. For example, heat-shock treatment of cells induces the expression of cytoprotective proteins. After exposure to a heat shock *in vitro*, embryonic stem cell–derived cardiomyocytes displayed an increased survival upon implantation. Other research demonstrated key proangiogenic and prosurvival functions of glucose in MSCs upon *in vivo* transplantation, proposing to integrate slow-releasable glucose inside the scaffolds to ensure better cell survival in constructs. On the other hand, some strategies aim to enhance the secretion of proangiogenic GFs, which then subsequently promote the neo-vascularization of the TE construct *in vivo*. For instance, the hypoxic pretreatment of cells can increment the secretion of proangiogenic factors (e.g., VEGF) by inducing the expression of the transcription factor hypoxia inducible factor 1, α .

14.5.3 Strategies to promote inosculation

The time frame of vessel prematuration depends on construct properties and cell types. For instance, HUVECs and MSCs coseeded in a polyurethane scaffold in the presence of PRP showed a spatial reorganization into tubular structures after 7 days of *in vitro* culture, as shown in laminin immunostaining of histological sections ([Fig. 14.8](#)). There, luminal structures could be followed over up to 80 μm . Upon implantation, inosculation between the preformed microvascular network and the host tissue can take hours to days, depending on the preformed vessels maturity (angiogenic activation) at the point of implantation as well as on the growth capacity of the vessels.

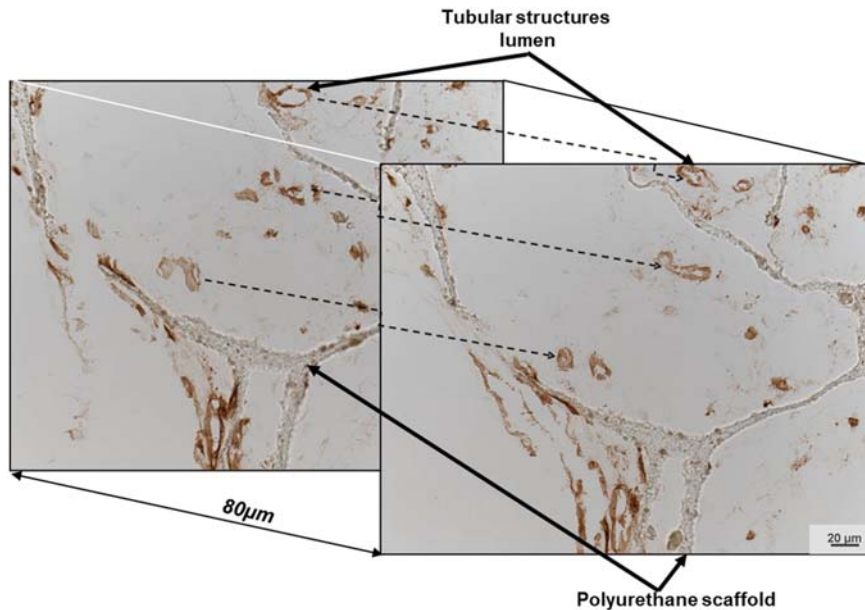


FIGURE 14.8

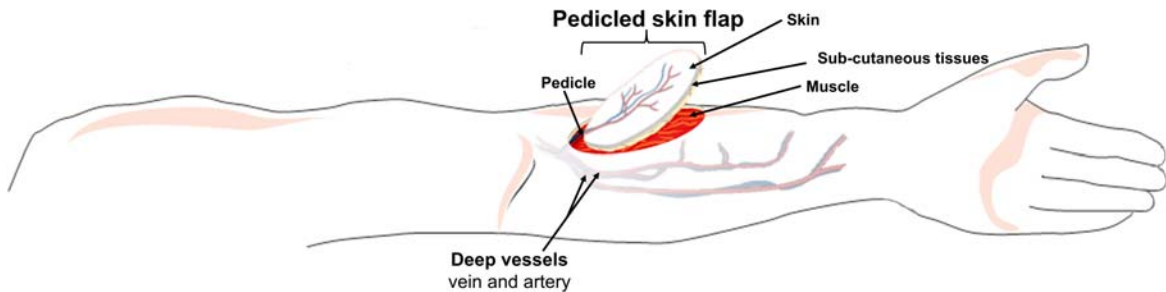
In vitro formation of luminal structures in precellularized 3D scaffold. Adapted from Duttenhoefer, et al. *3D scaffolds co-seeded with human endothelial progenitor and mesenchymal stem cells: evidence of prevascularisation within 7 days*. *Eur Cell Mater.* 2013;26:49–64.

To enhance the ischemic tolerance of cells during the initial stages after implantation, some possibilities can be envisaged. A construct combined with vascular pedicles can be directly anastomosed to the host microvasculature through microsurgery during implantation resulting in immediate restoration of blood perfusion.

Alternatively, the angiogenic activity of preformed microvascular networks can be optimized by preconditioning the implant in specific media or gels, to stimulate the cellular production of VEGF or the outgrowth of microvessels. Low oxygen or hypoxic cultures can also potentiate the angiogenic nature of cocultures for prevascularized constructs. When cultured in hyperconfluent conditions under hypoxia for up to 5 days, the adipose-derived SVF (containing adipose stem cells, endothelial progenitors, endothelial and hematopoietic cells, and pericytes) showed the formation of contiguous cell sheets with endothelial cells arranged in branched and highly complex tube-like structures, without the addition of extrinsic GFs.

14.5.4 In situ prevascularization

A major drawback of the aforementioned in vitro prevascularization strategies is the fact that they involve complex and time-consuming cell isolation, seeding, and cultivation procedures. In addition, if the seeding of scaffolds with cells or cell aggregates does not necessarily result in the development of functional microvascular networks, the increased regulatory requirements (GLP, GMP) make the translation of such strategies into clinical practice extremely challenging (see [Chapter 21](#) Clinical Translation). To overcome these problems, in situ prevascularization has been introduced in TE. In such a

**FIGURE 14.9**

Schematic representation of a vascular pedicle skin flap.

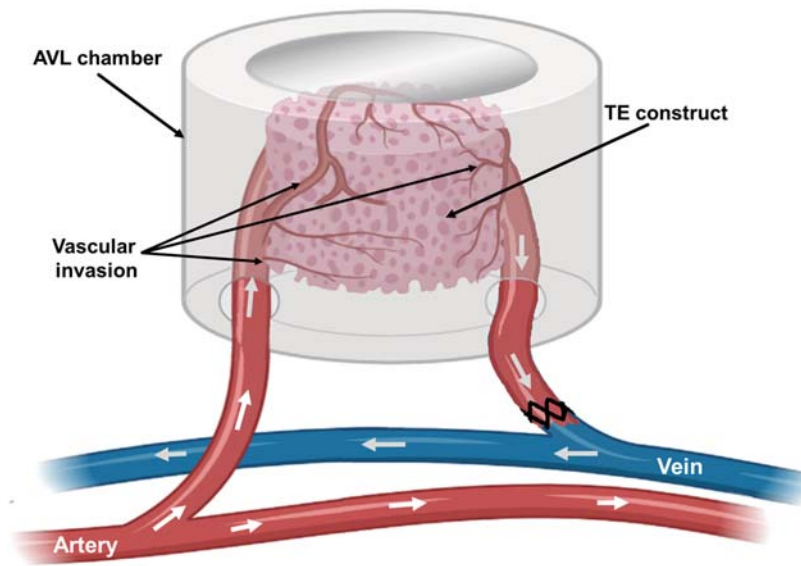
concept, the human body is used as a “bioreactor” to generate a microvasculature within tissue constructs in different ways. One technique already developed in the 70s is referred to as flap technique and consists of wrapping an implant inside a segment of tissue presenting an independent blood supply (Fig. 14.9). In case of the “vascular pedicle skin flap”, the flap is still linked to the donor site. On the opposite, in “free flap” the isolated tissue and its vasculature are detached from their site of origin and transferred to a new location with an intact vascular pedicle that will be surgically connected to the vasculature of the new site. However, requiring tissue transfer to another location, this method creates a second lesion in the body with all the associated potential risks.

Another approach is the implantation of scaffolds into an accessible and well-vascularized host site of the body, such as the subcutis or a superficial muscle. This induces an angiogenic host tissue response which includes the growth of functional blood-perfused microvessels that randomly integrate into the implant over time. Subsequently, these prevascularized scaffolds are excised and transferred to the final defect site. Until inosculation has taken place, the cells inside the implant suffer from hypoxia and parts of the construct may not survive this critical phase. Therefore, prevascularized tissue constructs may be alternatively generated with a vascular pedicle by flap fabrication and arterio-venous loop technique (Fig. 14.10).

These constructs can be microsurgically anastomosed to vessels at the implantation site, resulting in an immediate blood perfusion. Nonetheless, irrespective of the approach, all of these in situ prevascularization strategies require three surgical interventions, i.e., the implantation of scaffolds for prevascularization, their removal, and their final insertion into the host defect site. Hence, although highly promising and already tested under clinical conditions, in situ prevascularization needs to be simplified. One idea is to intraoperatively seed the scaffolds with isolated functional MVFs from adipose tissue and then directly implant them in the defect site in a one-step procedure. Under experimental conditions, these MVFs have already been shown to develop into fully functional microvascular networks inside scaffolds within a short time.

14.6 In vivo models

The approaches already described in previous paragraphs of this chapter require to be functionally validated in in vivo models, enabling the assessment of specific parameters, such as (1) the extent of vessel ingrowth into the TE constructs, (2) the survival level of the implanted cells inside the constructs, and (3) their functional capacities in vivo. This section describes examples of living models commonly used

**FIGURE 14.10**

Schematic representation of an arterio-venous loop chamber.

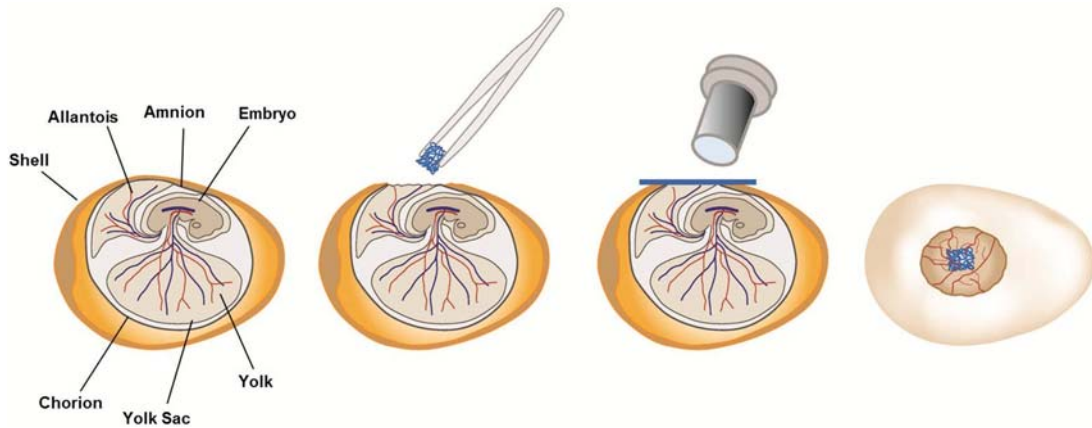
to obtain such information, with a focus on small animals, because of their easy access, low cost, and high throughput. Immune-incompetent animals allow investigating the performance of human cell-based TE constructs. **Athymic** nude mice/rats are T-cell deficient, whereas **severe combined immunodeficiency** (SCID) animals have deficiencies in both T- and B-cells as well as impaired natural killer cells. In such animals, the **xenogeneic** in vivo transplantation and testing of human cells is therefore possible with limited risk of rejection.

To test the vascular ingrowth, a well-vascularized bed with reproducible angiogenesis is needed. To assess the engraftment and the functionality of generated tissues, in vivo implantation can be performed **ectopically** or **orthotopically**. Ectopic implantation evaluates the evolution of constructs in a generic intravital environment (typically in subcutaneous locations), whereas orthotopic models are obtained by the implantation into environments similar to the tissue to be formed by the graft (in a skin defect for skin substitutes, for instance). Engraftment and formation of the desired tissue by the implanted cells can then be either monitored during the in vivo phase or examined after graft explantation (ex vivo).

14.6.1 Assessments of the vascularity within constructs—in vivo models

To assess the vascularization potential of TE constructs, two different models are commonly used.

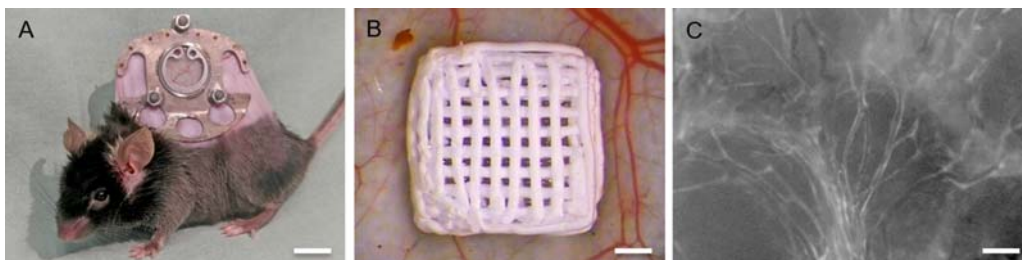
The chicken chorioallantois membrane (Fig. 14.11), often referred to as CAM, is a vascularized membrane of the chicken embryo reachable through the perforation of the eggshell. In this model, materials (like cell-based TE constructs but also tumors) can be physically applied and tested. The tissue around the implant can be monitored and the vascular growth analyzed. CAM is technically simple and

**FIGURE 14.11**

Schematic representation of the chorioallantois membrane procedure (CAM).

inexpensive, which allows for large-scale screening. Until day 10 of fertilization, it is not considered to be an animal model in many countries, which dramatically reduces ethical concerns and regulatory requirements. Mammalian xenografts can be implemented due to the lack of rejection by the host membrane. Even though the visualization of new vessel formation can sometimes be challenging, it also allows for noninvasive, nondestructive serial observations. Nevertheless, as it is a nonmammalian and embryonic model, in which angiogenesis occurs both by sprouting and **intussusceptive** (where one vessel splits into two) mechanisms, the significance of the obtained results must be carefully considered. In addition, this model exhibits nonspecific inflammatory reactions, poorly predictive of the mechanisms occurring in a more clinically relevant scenario.

The dorsal skinfold chamber is another model allowing for the assessment of vascularization within implanted scaffolds and tissue constructs. Here, two symmetrical titanium frames are implanted on the extended **dorsal skinfold**, pinching a double layer of skin (Fig. 14.12a). A circular area (15 mm in diameter) of one of the skin layers is excised. The remaining layer consisting of striated muscle, subcutaneous

**FIGURE 14.12**

Skinfold chamber. A: C57BL/6 mouse 48 hours after implantation of a dorsal skin fold chamber. Scale bar = 10 mm. B: Polyactic-co-glycolic acid (PLGA) scaffold within mouse dorsal skin fold chamber model. Scale bar = 0.5 mm. C: Intravital fluorescence microscopy (blue light epic-illumination, contract enhanced by 5% FITC-labeled dextran) of PLGA scaffolds on day 14 after implantation into the dorsol skinfold chamber. Scale bar = 65 μm .

tissue, and skin is therefore apparent, can be used as a target tissue for scaffold implantation (Fig. 14.12b), and is covered by a coverslip incorporated into the observation window of the titanium frame. Thereafter, **intravital** fluorescent staining coupled with multifuorescence microscopy and image analysis allows for the accurate quantification of parameters such as: i) angiogenic sprouting and network formation, ii) microvessel density, and iii) microhemodynamics. (Fig. 14.12c). Specific immuno-staining also permits the study of cellular functions. This model is available in immune-competent rats, hamsters, and mice as well as in nude and SCID mice. It is a perfect model to study the early, host-driven inflammatory and angiogenic responses to implanted materials and its design allows one to modulate specific parameters in the chambers, like temperature or composition of the surrounding milieu. The major limitations related to this model are that it only allows for the testing of rather small implants (not exceeding $3 \times 3 \times 1$ mm), and that it is not suitable for long-term studies (2–3 weeks maximum).

The above-described methods enable continuous monitoring of in vivo vascularization within a limited time frame, whereas models for long-term studies are presented below.

Subcutaneous, ectopic implantation in mice or rats: In this model, the animal is anesthetized and an incision in the skin (typically not longer than 15 mm) is made by using a surgical knife or scissors under sterile conditions. The TE construct is then **subcutaneously** introduced, and the incision is closed. The duration of the in vivo implantation is highly variable, according to the aim of the experiment. Short times (e.g., a few days to 2 weeks) are sufficient to assess the initial vascularization and anastomosis of prevascularized grafts, whereas the study of the formation and maturation of the target tissue may require short (for skin TE constructs) to long implantation time (for tissues like bone). After excision, the constructs can be analyzed for specific features by applying various techniques, some of which are described in more detail below. Overall, subcutaneous implantation is a technically simple, informative, and inexpensive in vivo approach. Moreover, even if it is a rather time-consuming approach and the subcutis is not a very relevant site for engraftment and development of specific tissues, the subcutaneous implantation allows for the assessment of the intrinsic performance of a graft in a standardized environment and independently of the influence of trophic factors present in the specific target tissue.

By incorporating the TE construct directly into its target tissue, orthotopic implantation offers a more physiological but also much more clinically relevant model for translational strategies. It allows for the actual testing of the graft functionality in the physiological or pathological context of its target environment. There are as many models as there are species (from mouse to goat or horse), tissues, and pathologies.

14.6.2 Assessment of graft vascularization and functionality of engineered tissues—in vivo imaging

Several in vivo imaging techniques allow for the noninvasive real-time monitoring of transplanted cells as well as the overall vascularization of implanted scaffolds. Certain techniques have the intrinsic ability to visualize blood flow and depict the perfusion of the constructs, whereas labeling the cells with reporter molecules (termed **contrast agents**) before their implantation allows one to identify and surveil them over time. The technologies for vascular imaging can be classified into three major groups: non-optical, optical, and hybrid methods (Table 14.3). Among the nonoptical approaches, Magnetic Resonance Imaging (MRI) can track cells through contrast agents, providing information on their position

Table 14.3 Main imaging techniques for in vivo assessment of vascularization and their properties.

	Imaging technique	Contrast	Resolution (μm)	Penetration depth (mm)
Nonoptical	Magnetic resonance	Nuclear resonance	100–500	Whole body
	Ultrasound	Acoustic reflectivity	30	150
	Computed tomography (VCT)	Radiocontrast	50–200	Whole body
	Microcomputed tomography (μCT)		30–200	Whole body
	Positron emission tomography	Radioactive emission	1000–2000	Whole body
Optical	Optical coherence tomography	Optical scattering	4–10	1–3
	Single-photon microscopy	Reflectivity, molecular spectral responses	0.3	0.2
	Two-photon microscopy	Specific nonlinear optical properties	0.3–0.7	0.3–1.6
	Laser speckle contrast imaging	Speckle contrast	10	0.5
	Orthogonal polarization spectral imaging	Reflectivity	1	1
Hybrid	Photoacoustic tomography	Spectral absorption, thermoacoustic properties	50–800	0.1–50
	Photoacoustic microscopy		0.1–100	15–50

and functions as well as on the time point of cell death. Moreover, a technique named MRI-assisted angiography enables the examination of vascular health in a whole-body setting, with or without contrast agents (via arterial spin labeling). The vascularization can also be monitored by Computed Tomography (CT) upon systemic application of vital radio-opaque and radio-dense contrast agents or by Ultrasound Imaging (UI), which measures hemodynamic information, such as blood perfusion of the microvasculature growing into the implant site. Specifically, the Doppler UI elicits the visualization of local fluid flows and, when combined with exogenous contrast agents (i.e., microbubbles), can also reveal microvessel networks with enhanced sensitivity, determine the blood flow rates, and measure both the volume and size of capillaries. Due to the poor spatial resolution or contrast, nonoptical methods have been predominately confined to macrovascular imaging, whereas optical techniques are effective for microvascular imaging.

Multiscattered optical imaging (like diffuse optical tomography) presents a penetration depth of a few centimeters into tissues but displays poor spatial resolution. Other optical methods (single-photon and two-photon fluorescence microscopy, laser speckle contrast imaging, orthogonal polarization spectral imaging, and optical coherence tomography) display higher resolution but at the same time limited penetration depths (~ 0.2 – 1.5 mm). Finally, combining high optical absorption contrast with high ultrasound resolution, photoacoustic imaging (PAI) is a hybrid technology providing label-free vascular imaging with a scalable spatial resolution (0.1 – 800 μm) and penetration depth (0.1 – 50 mm). In PAI,

the blood hemoglobin absorbs short laser pulses and generates ultrasonic waves, which are then detected and used to develop a picture in coregistration with UI.

14.6.3 Assessment of graft vascularization and functionality of engineered tissues—ex vivo analysis

Several ex vivo methods to identify implanted cells and discriminate them from the host-derived infiltrating cells are also available. For example, vascular cells can be labeled prior to their implantation with cell-staining dyes, such as carboxy-fluorescein succinimidyl ester or the lipophilic fluorescent dye Dil. Moreover, when human vascular cells are implanted into immune-incompetent mice or rats, they can be differentiated from the host ones based on the different species of origin. In situ hybridization for species-specific genomic motifs, like the human **ALU sequences** or the murine **SINE elements**, enables recognition of the origin of the cells. This technique can be used to determine which cell species contributed to the tissue and vascularity present in the explanted graft. Alternatively, immune-histochemical staining using antibodies directed against human-specific or murine-specific epitopes of vascular-specific proteins, like CD31 or CD34, reveals the specific contribution of implanted and host cells to the generation of blood vessels in the grafts.

14.7 Translation into clinics

The clinical success of TE constructs is so far limited to thin tissues, such as skin, cornea, large diameter blood vessels, bladder, and avascular tissues like cartilage (see [Chapter 21 Clinical Translation](#)). For more complex tissues and organs, such as bone, kidney, or liver, which are highly organized and composed of numerous cell types, clinical applications of TE substitutes are still limited mostly to pre-clinical models and Phase I clinical trials. Despite the critical advances in the strategies to improve the vascularization of clinically sized constructs described in this chapter, efficient vascularization of complex grafts and engineered organs remains a very challenging issue on the way to routine organ regeneration. Here, we described strategies aiming to promote neo-vascularization and accelerate vascular ingrowth into engineered grafts. In other approaches, the need for immediate vascularization of the graft is skirted by engineering grafts resistant to this initial **hypoxia**. Alternative strategies, not described here, rather rely on the use of medical devices containing functional cells, such as pancreatic cells to treat diabetes, or cell-free mechano-prostheses, such as artificial hearts.

TE can synergize with surgical techniques to de novo fabricate artificial flaps, pedicled grafts, and organs by combining fully engineered tissue with arteriovenous bundles or loops. Such “regenerative surgery” paradigms require a complex interplay among biologists, engineers, material scientists and clinicians, and need to be implemented into Phase I/II clinical trials to provide proof-of-concepts demonstrating their safety and efficacy.

The need for TE constructs, or tissue substitutes, is usually related to the size of the defect to be healed and therefore requires large 3D volumes. After the proof-of-concept is obtained in vitro and in small animal models, an upscaling of substitute grafts is mandatory and requires actual validation in large animals to justify their potential use in the clinic. Proper systems (e.g., bioreactors, see [Chapter 13 Bio-reactors: enabling technologies for research and manufacturing](#)) that will be organ-specific (in terms

of the mechanical and biological environment) and generate large scale and/or tailored engineered tissues still need to be developed. Issues related to cost-effectiveness, standardization of some of the procedures, and quality management are also among the many hurdles still hindering the routine clinical application of prefabricated grafts (see [Chapter 20 Product and process design: toward industrial TE manufacturing](#)).

Summary

- Neo-vascularization engineered tissues is required to ensure a sufficient supply of oxygen and nutrients and the removal of metabolites.
- In adult life, neo-vascularization is mainly driven by angiogenesis, a highly regulated, multi-step process driven by proangiogenic growth factors and cell–cell interactions.
- The microstructure of an engineered tissue construct, particularly its porosity, can guide and promote the neo-vascularization of the construct.
- Biofabrication techniques, such as 3D printing, can be applied to generate constructs with a complex vessel system.
- Vascular sprouting and invasion of endothelial cells in a tissue construct can be induced by incorporation of proangiogenic factors in the construct.
- Incorporation of mature endothelial cells, EPCs, or MVFs may be used to promote intrinsic neo-vascularization.
- In vitro prevascularized constructs can be produced by vascular and tissue-specific cells coseeded into scaffolds, aggregated into multicellular spheres, or layered into cell sheets.
- Allogenic decellularized tissues retain the natural architecture of the extracellular matrix and can be used to guide neo-vascularization.
- In situ prevascularization techniques aim to use the human body as a bioreactor to promote neo-vascularization in a tissue-engineered construct.
- Various preclinical models have been developed to assess the vascularity of grafts, including the chorioallantois membrane model, the dorsal skin-fold chamber, and the ectopic subcutaneous or orthotopic implantation of the construct in mice or rats.
- Modern imaging techniques allow to closely monitor neo-vascularization processes in vivo and ex vivo.
- The potential translation of strategies to clinical applications must be considered, with particular attention for minimal manipulation approaches, scaling-up possibilities and cost-effectiveness.

Classical experiment

Use of hypoxia-resistant tissues to overcome the limitations of delayed vascularization: The example of endochondral ossification

Mesenchymal stem/stromal cells (MSCs) are typically used to generate bone tissue by a process resembling intramembranous ossification, i.e., by direct osteoblastic differentiation. This process is known to strongly rely on a rapid vascularization of the implanted MSC-containing graft in vivo. However, most bones develop by

endochondral ossification, via remodeling of hypertrophic cartilaginous templates. A major advantage of this ossification route is that the implanted construct is made of cartilage, a tissue known to require very low oxygen tensions in a physiological situation, the cellular part of which can therefore survive for prolonged periods in hypoxic conditions before vascular ingrowth at the site of implantation can take place by angiogenic processes from the surrounding tissues.

Classical experiment—continued

In a study by Scotti and colleagues, the capacity of expanded human MSCs to generate bone tissue through such an endochondral ossification process was tested. To mimic condensation and chondrogenic differentiation of mesenchymal cells typical of endochondral ossification, human bone marrow MSCs were expanded in monolayer cultures and thereafter 5×10^5 cells/insert were cultured in a transwell, scaffold-free system for 1 or 2 weeks in a chondrogenic medium containing TGF β 1 (prechondrogenic and early hypertrophic stages, respectively), or for 3 weeks in chondrogenic medium followed by 2 weeks in a hypertrophic medium (without TGF β 1 and with beta-glycerophosphate and thyroxine; late hypertrophic stage). The resulting tissues in the form of disks show increasing degrees of chondrogenic differentiation and hypertrophy.

When implanted subcutaneously in nude mice and retrieved after 4 or 8 weeks, the implanted templates exhibited an organized series of events. A cellular condensation and hypertrophic chondrogenesis induced the

formation of a bony collar by perichondral ossification. Matrix remodeling by matrix metallo-proteases and osteoclastic activity took place and paralleled the vascular ingrowth from the host tissue into the cartilaginous template by angiogenesis. Indeed, capillary vessels positive for CD31 (PECAM-1) were shown to invade the graft starting from the outer matrix, likely to transport host osteoclasts, nutrients, and proapoptotic signals to the internal hypertrophic cartilaginous template (Fig. 14.13). In the vascularized regions of the graft, bone matrix deposition onto the resorbed cartilaginous template could take place and bone tissue was formed, including functional hematopoietic foci, suggesting the formation of a functional bone organ.

The documented endochondral morphogenesis, with essential features of physiological endochondral ossification, is an example of how tissue engineering could harness developmental processes to overcome limitations related to delayed vascularization.

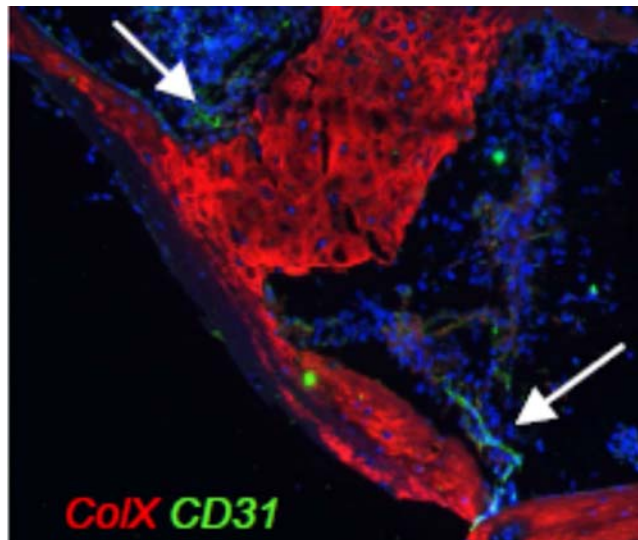


FIGURE 14.13

CD31⁺ endothelial cells (green on the picture) of ingrowing vessels, penetrating the hypertrophic matrix, which contains collagen type X (ColX) toward the center of the graft. Those blood vessels are located right next to the cartilaginous areas, which undergo remodeling (arrows). From Scotti, et al. *Recapitulation of endochondral bone formation using human adult mesenchymal stem cells as a paradigm for developmental engineering*. Proc Natl Acad Sci USA. 2010;107(16):7251–7256.

State-of-the-art experiment

Improvement of islet transplantation by the fusion of islet cells with functional blood vessels

In the treatment of type 1 diabetes mellitus, the transplantation of pancreatic islets (PIs) constitutes a promising therapeutic strategy. To achieve a recipient's insulin independency, many islets and therefore multiple islet donors are required. The success of such a procedure depends on both donor availability and islet tissue integration after transplantation. Upon isolation, the dense microvascular networks of the islets are stripped away, leading to insufficient neo-vascularization following implantation and engraftment failure.

Hence, to promote the prevascularization of isolated islets, they are usually subcutaneously implanted or combined with other cell types such as endothelial cells prior transplantation. However, the formation of functional microvascular structures from single cells is a long process that can take up to 10 days. Being functional luminal microvascular units, MVFs represent a promising alternative to achieving a sufficient prevascularization of islets. Such an approach has been described by an experimental study of Nalbach et al. (EMBO Molecular Medicine in 2021), which focuses on the prevascularization of islet organoids by means of a fusion of PI cells and MVFs (Fig. 14.14). After 5 days of in vitro coculture of 2,000 PI with 200 MVFs, the authors observed the formation of vascularized islet

organoids (PI + MVF), which showed comparable properties (size, cell contents, insulin secretion) when compared to freshly isolated islets (FIs) or cultured islets (CIs), whereas the cellular viability was slightly higher in PI + MVF. Extended in vitro analysis further confirmed the functionality of the pancreatic cells, as indicated by both the expression and secretion of insulin. Compared to FI or CI, PI + MVF revealed a strong angiogenic potential and vascularization capacity. PI + MVF further showed both rapid reorganization of individual MVFs into a dense microvascular network and a higher number of proliferative endothelial cells in vitro. Upon subcutaneous implantation (skinfold chamber model), PI + MVF revealed both faster revascularization and interconnection of MVFs with the microvessels of the host tissue as well as a higher number of larger microvessels when compared to FIs or CIs. When implanted in a diabetic mouse model, animals receiving 250 PI + MVF showed an improved restoration of normal glycemia, thus insulin independence 7 days after transplantation. Of interest, animals receiving an identical number of FIs did not.

This study represents a breakthrough, knowing that in actual clinical settings islets from at least four different donors are required to reach insulin independence and the overall success of the procedure strongly relies on the engraftment of those islets.

State-of-the-art experiment—continued

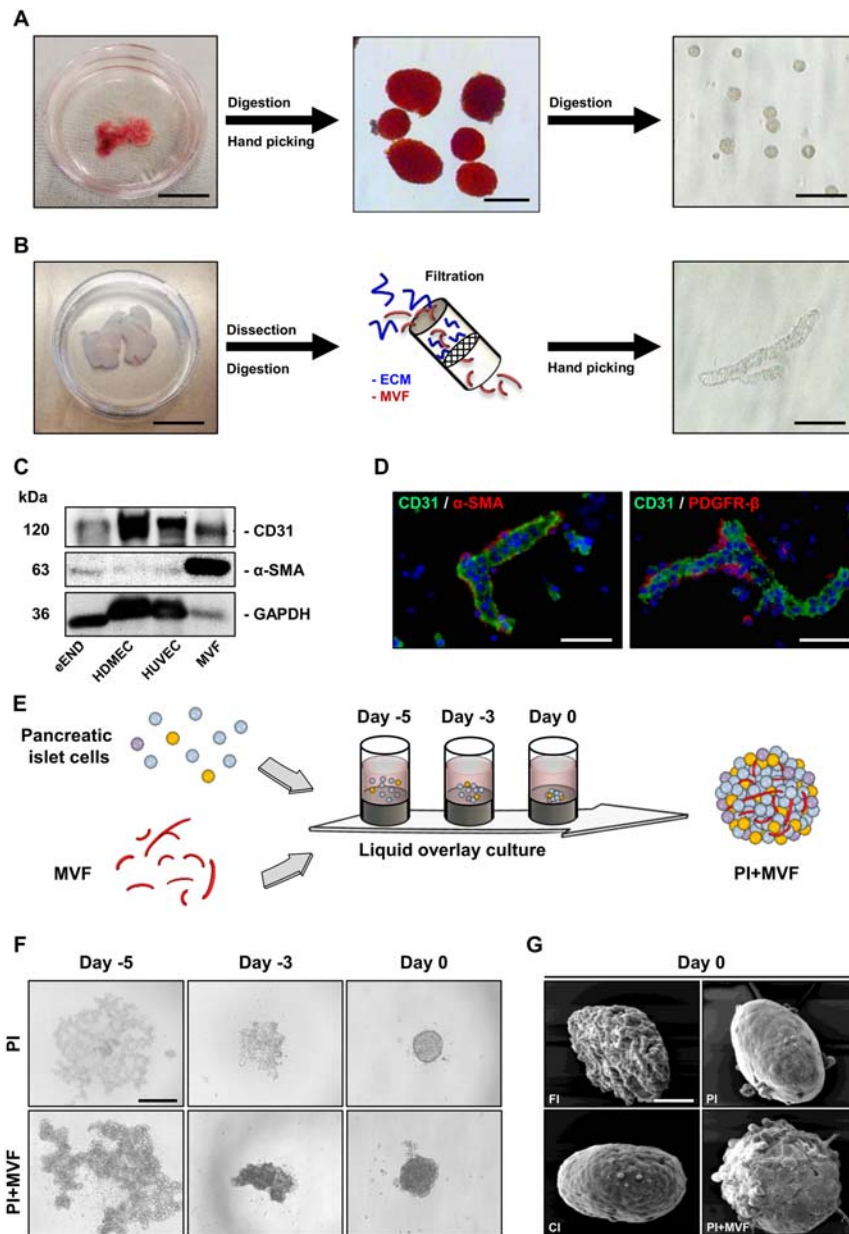


FIGURE 14.14

(a) Mouse pancreas is excised and digested with collagenase. Islets are then separated by hand picking and further digested into single islet cells. Scale bars: 10 mm (*left image*); 200 μ m (*middle image*); 60 μ m (*right image*). (b) Epididymal fat pads are isolated and processed mechanically and enzymatically. The obtained mixture, containing MVFs and ECM, is filtered for the purification of MVFs. Scale bars: 20 mm (*left image*); 25 μ m (*right image*). (c) Western blots of CD31, α -SMA, and GAPDH in whole cell extracts of eENDs, HDMECs, HUVECs, and MVFs. (d) Immunofluorescence staining of CD31/ α -SMA and CD31/PDGFR- β in MVFs. Cell nuclei are stained with Hoechst 33342 (*blue*). Scale bar: 20 μ m. (e) Schematic illustration of PI + MVF generation. By means of liquid overlay technique, 2,000 pancreatic islet cells and 200 MVFs are cocultured for 5 days. (f) PI and PI + MVF on days 5, 3, and 0. Scale bar: 200 μ m. (g) Surface morphology of FIs, CIs, PIs, and PI + MVF on day 0 as visualized by scanning electron microscopy. Scale bar: 50 μ m. From Nalbach, *et al.* *Improvement of islet transplantation by the fusion of islet cells with functional blood vessels*. EMBO Mol Med. 2021.

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14.9 Assessment of your knowledge

- (a) Answer the following questions to assess your command of terminology, facts, concepts, and theories learned in this chapter:
 1. External inosculation is a process where:
 - a. an autonomous vascular network develops within the implanted construct
 - b. a preexisting vascular network develops interconnections with the host microvasculature outside the space of the implanted construct
 - c. a vascular network fully surrounds the space of the implanted construct
 - d. vascular cells develop vessel structures out of their physiological environment
 2. The detection of ALU sequences is used to discriminate cells
 - a. of endothelial origin
 - b. of ectodermal origin
 - c. of human origin
 - d. in quiescent state
 3. Platelet-rich plasma is
 - a. isolated from blood samples
 - b. an expensive biomaterial for growth factors provision
 - c. a cost-effective biomaterial with a well-standardized composition
 - d. shows a reduced donor-to-donor variability
 4. Hypoxic preconditioning of cells can be used to:
 - a. decrease the metabolism of cells before implantation
 - b. potentiate the angiogenic nature of cocultures and increment the secretion of proangiogenic factors
 - c. promote the occurrence of necrosis at the scaffold borders
 - d. downregulate the expression of the transcription factor hypoxia-inducible factor 1, alpha (Hif1 α)
 5. The CAM:
 - a. is technically simple but expensive
 - b. can only be used for studying nonmammalian xenografts because of its nonspecific inflammatory reactions
 - c. understates limited ethical regulation as compared to other animal models
 - d. does not allow for large screenings
 6. Intussusceptive angiogenesis occurs
 - a. when new vessels arise from previous vascular loop-shape networks
 - b. only if vascular cells are biochemically preconditioned in vitro

- c. in early stages of the neo-vascularization process
- d. when a new blood vessel is created by splitting of an existing blood vessel in two.
- 7. Endothelial progenitor cells have been explored as angiogenic cell source. Which statements are correct?
 - a. Endothelial progenitor cells show greater proliferation capacity compared to mature endothelial cells.
 - b. Endothelial progenitor cells show lower proliferation capacity compared to mature endothelial cells.
 - c. Endothelial progenitor cells can be isolated from peripheral blood.
 - d. The prime function of endothelial progenitor cells is to support sprouting angiogenesis.
- 8. Mural cells are often applied in combination with an endothelial cell source. What is the rationale behind this?
 - a. Mural cells prevent a too fast formation of vascular sprouts.
 - b. Mural cells support the sprouting process, stabilize newly formed vessels, and guide their maturation.
 - c. Mural cells secrete proangiogenic factors and guide new vessel formation by building gradients of these factors.
- 9. Photoacoustic imaging is:
 - a. An optical technique for angiogenesis visualization
 - b. A technique purely applied to excised tissues
 - c. A purely optical technique for visualization of blood flow
 - d. A hybrid technique revealing the photo-absorption of hemoglobin
- 10. The orthotopic model:
 - a. Allows assessing graft functionality independently of the influence of tissue-specific biochemical factors.
 - b. Allows assessing graft functionality within the patho-physiological context of its target implantation site.
 - c. Is exclusively generated into small animals.
 - d. Is obtained by creating subcutaneous pockets into immune-deficient animals.
- 11. Microvascular fragments (MVs) are suggested as vascularization units for tissue engineering. Why are they superior when compared to single cells?
 - a. MVs already represent biologically intact vessel segments with a physiological tube-like structure.
 - b. After their seeding onto biomaterials/scaffolds, MVF can bridge relatively wide distances due to their lengths, leading to rapid creation of a mature microvasculature.
 - c. After their isolation, MVFs contain stem cells within their physiological niches.
- 12. The dorsal skinfold chamber model is frequently used for assessing the vascularization of scaffolds implanted in vivo. What makes this model special?
 - a. It allows continuous access to the implant through the observation window.
 - b. It allows studying the individual cell types by means of specific immuno-staining approaches.
 - c. The size of the skinfold chamber can easily be adapted and used for all sizes of rodents.
 - d. It can be used to even analyze large scaffolds up to multiple centimeters.

13. In vitro prevascularization generally involves the combination of:
 - a. Growth factors and endothelial cells
 - b. Tissue-specific cells and growth factors
 - c. A vascular tree and proangiogenic factors
 - d. Endothelial cells and tissue-specific cells
 14. Cell survival inside an engineered tissue upon in vivo implantation can also be improved by:
 - a. A preconditioning of the tissue with gamma irradiation
 - b. The use of a material inducing inflammation of the host tissue
 - c. Incorporation of glucose inside the construct
 - d. The use of immortalized cells
 15. Endochondral ossification occurs through:
 - a. A direct differentiation of mesenchymal cells in bone-forming cells
 - b. The generation of a cartilage template that gets remodeled to bone upon vascularization
 - c. Endothelial cell differentiation into boneforming cells
 - d. Differentiation of osteoclasts into bone-forming cells
 16. Decellularized matrices
 - a. Are made of synthetic polymers
 - b. Are the result of chemical and enzymatic treatments of natural tissues
 - c. Can be produced using 3D printing technology.
 - d. Can only be produced from bone and cartilage.
 17. What is a typical method to assess the angiogenic potential of cells?
 - a. An MTT assay.
 - b. The assessment of network formation on Matrigel.
 - c. Measurement of collagen deposition.
 18. What might be the challenges for the clinical application of angiogenic cells?
 - a. The heterogeneity of EPC populations.
 - b. EPC cannot be obtained in an autologous setting.
 - c. The lack of established characterization methods.
 19. What are the important characteristics of a scaffold for a successful neo-vascularization:
 - a. The presence of interconnected pores
 - b. Its price and available size
 - c. Its tubular shape
 - d. Its micro/macro topography
 20. Is it possible to develop a “universal” proangiogenic scaffold for all tissues?
 - a. Yes, as soon as it promotes vascularization.
 - b. Yes, vessels are the same everywhere.
 - c. No, microvessels from different tissues have different structures/components.
 - d. No, the scaffolds also need to fulfill the specific requirements of the target tissue.
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real-life, clinical, and scientific situations.

1. Describe the advantages and disadvantages in using ectopic and orthotopic animal models to study vascularization of tissue constructs.
2. Describe the main types of imaging used for the assessment and monitoring of the implant vascularization in vivo.
3. Describe at least three methods to promote tissue-engineered construct vascularization, including advantages and disadvantages.
4. What are key factors to promote the neo-vascularization of constructs and why?
5. Describe the vascular tree.
6. Describe the main strategies to modify the physico-chemical properties of scaffolds to enhance their vascularization.
7. Describe the functional characteristics of endothelial cells.
8. Can different vascularization strategies (prevascularization, proangiogenic materials, in situ vascularization) be combined? Imagine and describe a possible tissue product based on a such strategy.
9. What are the advantages of decellularized tissues? Please describe a concrete example.
10. Discuss why the lack of vascularization is a problem and give two possible strategies to solve it.

Challenge-based learning

Perfusion of a tissue-engineered islet of Langerhans

Note for teachers: a challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

The survival and function of organs and tissues rely on the constant irrigation and functional vascularization to provide oxygen and nutrients through arteries, and to remove toxins and metabolic by-products through veins. Neo-vascularization in embryological tissues is always on par with tissue growth: the bigger the tissue, the more vascularized it is. This biological design requirement successfully bypasses the limitation of simple diffusion of nutrients and oxygen through tissues. It is well known and documented that tissue diffusion is limited to 200 μm . The long-term vision is to obtain a fast and cost-effective strategy to vascularize engineered tissues to ensure their survival and function after implantation.

Motivation and stakeholders

Patients suffering from type-I diabetes require daily insulin injections to control hyperglycemia after a meal. Constant glucose monitoring and insulin injections represent the

burden for both patients and caretakers. Tissue engineering strategies are underway to generate functional islet of Langerhans-like organoids that can release insulin upon implantation. The islet-like organoids can be fabricated with iPSCs, which are a relevant cell source in modern tissue engineering and should house functional vascularization for the systemic release of insulin. These organoids should sense blood glucose levels, anastomose with the host vasculature, and release insulin on demand. Solutions to include vasculature into tissue engineered islets of Langerhans should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders, such as diabetes patients, internal medicine doctors, pediatricians, nutrition experts, biofabrication engineers, iPSC biologists, and biomedical engineers.

Problem definition

Currently, the functional cell types of a prevascularized islet of Langerhans organoid can be obtained by differentiation of iPSCs. However, maturation of the blood vessels and sufficient nutrient supply of the tissue requires that they are perfused during the in vitro culture stage. Therefore, a fully vascularized islet of Langerhans needs to be engineered to be used in patients suffering from type-I diabetes.

Challenge-based learning—continued

Challenge

To design a strategy to fabricate islet of Langerhans organoids with functional vasculature to replace continuous insulin injections in type-I diabetes patients.

Learning framework

Reading the Vascularization chapter and related literature will help you to understand the following:

1. The anatomy and physiology of islets of Langerhans.
2. The anatomy and physiology of blood vessels.
3. Physiological requirements of anastomosis.
4. Different strategies available to prevascularize tissues in vitro.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

5. Current protocols to differentiate iPSCs into the functional cells of a prevascularized islet of Langerhans.
6. Biomaterials used to engineer prevascularized islets.
7. Assays used to demonstrate islet and blood vessel functionality.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

14.10 Glossary

ALU sequences are the most abundant transposable DNA elements, containing over one million copies dispersed throughout the human genome.

Anastomosis is the connection between two blood vessels that are normally separated, diverging, or branching.

Angiogenesis is the growth of new capillaries from pre-existing blood vessels.

Athymic means lacking a normal thymus gland, resulting in a defective immune system.

Bioinks are biomaterials used to produce engineered/artificial live tissue using 3D printing.

Cell sheets are cells cultured as confluent layers along with their deposited extracellular matrix.

Contrast agents are substances used to increase the contrast of structures or fluids within the body in medical imaging.

Dorsal skinfold chamber is a rodent model for noninvasive microcirculatory analyses of striated muscle and skin tissue throughout an observation period of 2–3 weeks.

Ectopically refers to an abnormal location or position of something, e.g., an organ, a body part or the site of protein expression.

Endothelial cells are of mesodermal origin and form the lining of the blood vessels.

Endothelial progenitor cells are undifferentiated cells that can undergo endothelial differentiation and thus play roles in the regeneration of the endothelial lining of blood vessels.

Ex vivo refers to experimentation or measurements done outside the body in or on freshly isolated tissues.

Fenestrated (Fenestrae Latin for windows) are orifices or pores of 60–80 nm in diameter for the exchange of nutrients, waste products, and other substances, as seen in endothelial cells.

Fibrous encapsulation is the formation of scar tissue on the surface of an implanted biomaterial produced by myofibroblasts, as a consequence of incomplete phagocytosis.

Flap technique is a technique in plastic and reconstructive surgery where any type of tissue is lifted from a donor site and moved to a recipient site with an intact blood supply.

Hypertrophy is the increase in the size of cells.

Hypoxia is a condition in which the body or a region of the body is deprived of adequate oxygen supply at the tissue level.

Induced pluripotent stem cells are a type of pluripotent stem cells generated directly from a somatic cell by expressing the transcription factors Oct 4, Sox2, Klf4, and c-Myc.

Inosculation refers to the junction or connection of natural components, such as blood vessels, channels, or passages, to form a unity. Same as anastomosis.

Interconnectivity refers to an open connection between two adjacent pores of a biomaterial scaffold.

Intravital imaging is a form of microscopy that permits the observation of biological processes in living animals at a high resolution, enabling the distinction between individual cells of a tissue.

Intussusceptive is a process where a new blood vessel is created by splitting an existing blood vessel in two.

Lithography is a patterning process where design is etched onto a flat surface.

Luminal structures are the most internal layer of a tubular structure, such as an artery or intestine.

Matrices are noncellular materials in between cells of different tissues.

Matrigel is the trade name for the solubilized basement membrane matrix secreted by Engelbreth–Holm–Swarm mouse sarcoma cells.

Mechanobiology focuses on how physical forces and changes in the mechanical properties of cells and tissues contribute to development, cell differentiation, physiology, and disease.

Microvascular endothelial cells are endothelial cells isolated from small blood vessels in several tissues, such as skin or adipose tissues.

Microvascular fragments are vessel fragments isolated from adipose tissue that exhibit high angiogenic activity and represent a rich source of mesenchymal stem cells.

Mural cells are the vascular smooth muscle cells, and pericytes, of the microcirculation. Both types are in close contact with the endothelial cells lining the capillaries and are important for vascular development and stability.

Neo-vascularization is the de novo formation of new blood vessels.

Orthotopically refers to something that occurs in the usual place in the body.

Paracrine is a type of cellular communication in which a cell produces a signal to induce changes in neighboring cells, altering the behavior of those cells.

Pericytes are a type of perivascular cells, defined as multifunctional mural cells of the microvasculature, that wrap around the endothelial cells that line the capillaries throughout the body.

Perivascular cells are cells that provide stability and functionality to blood vessels and include vascular smooth muscles and pericytes.

Plasma-gas treatment is a process by which gasses (such as oxygen, argon, dry air) are used to etch a biomaterial to modify the physicochemical characteristics of its surface, facilitating the adsorption of biomolecules or the covalent grafting of active peptides.

Plasma-spray is a protective coating process in which melted (or heated) materials are sprayed onto a surface.

Platelet-rich plasma (PRP) is a concentrated preparation of platelets derived from whole blood after red blood cell removal.

Preconditioning is a concept, in which an entity is exposed to stress or stimulus in order to prepare that entity to be more resilient against the stimulus when and if the stimulus is encountered in the future.

Proteases are enzymes that break down proteins into smaller polypeptides or single amino acids by cleaving the peptide bonds within proteins.

Severe combined immunodeficiency (SCID) is a group of rare disorders caused by mutations in different genes involved in the development and function of T and B immune cells. Individuals with SCID appear healthy at birth but are highly susceptible to severe infections.

SINE elements are short repetitive, noncoding sequences ranging in size from 100 to 600 base pairs, widely distributed in eukaryotic genomes and have roles in genome organization, genome evolution, and modulating gene expression.

Smooth muscle cells are a specific type of muscle cells that are spindle-shaped which can tense and relax slowly and automatically because of the presence of myosin and actin proteins.

Spheroids are a type of three-dimensional cell modeling that better simulate a live cell's environmental conditions compared to a two-dimensional cell model, specifically with the reactions between cells and the reactions between cells and the matrix.

Stromal vascular fraction is a heterogeneous collection of cells contained within adipose tissue.

Subcutaneously means that an entity is located under the skin.

Surface topography refers to the three-dimensional features of a surface.

Vasculogenesis is the process of blood vessel formation in the embryo, occurring by the differentiation of mesenchymal cells into endothelial cells.

Wettability is the ability of a liquid to maintain contact with a solid surface, resulting from intermolecular interactions between the liquid and the surface.

Xenogeneic is a tissue or organ that is derived from, originating in, or being a member of another species.

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Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Skin tissue engineering and keratinocyte stem cell therapy

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15.1 Learning objectives

After reading this chapter, you will be able to:

- Describe the structure and function of skin and role of differentiation markers.
- Demonstrate understanding of the basic concept of wound healing and differences between acute and chronic wound healing.
- Demonstrate understanding of the concept of keratinocyte cell expansion in vitro and the role of keratinocyte stem cells and other adult stem cells for clinical application.
- Demonstrate understanding of the significance of dermis for keratinocyte grafting.
- Discuss the different types of tissue-engineered skin products and their role in wound therapy and future development.

This chapter is dedicated to the memory of Professor Harshad A. Navsaria, who passed away in 2014. Professor Navsaria, who was instrumental in the field of tissue engineering for skin transplantation, initiated the first edition of this book chapter.

Skin bioprinting technology has huge potential to facilitate fabrication of physiologically-relevant tissue and enable better and more consistent functional outcomes in burn patients. The use of bioprinting for skin reconstruction following burns is promising, and bioprinting will enable accurate placement of all the different native skin cell types and precise and reproducible fabrication of constructs to replace injured or wounded skin.

M. Varkey, 2019.

The regenerative medicine revolution is upon us. Like iron and steel to the industrial revolution, like the microchip to the tech revolution, stem cells will be the driving force of this next generation.

C. Hildreth, 2018.

15.2 Introduction

The **integumentary system** comprises the skin and its associated **appendages**, which include hair, nails, and exocrine glands (sebaceous and sweat glands). The skin is composed of the **epidermis**, **dermis**, and

subcutaneous tissue and is the largest organ of the body with a diverse array of functions including being a sensory and thermoregulatory organ and acting as a barrier for protection. Due to being exposed to biological, chemical, and UV insults, the skin is susceptible to injury, and therefore **wound healing** is vital for survival. Skin wound healing is an organized and complex set of processes, which results in the restoration of tissue structure integrity and tissue functionality. Wound healing falls into four sequential but overlapping phases that include **coagulation**, **keratinocyte**, **fibroplasia**, and **extracellular matrix (ECM) remodeling**¹ (Table 15.1). All these processes are coordinated by intracellular and intercellular signaling pathways and a variety of growth factors and cytokines that are released in the wound in a timely and orderly manner and play a role in cell recruitment, migration and proliferation, angiogenesis, reepithelialization, and ECM production. Wound healing in adult mammals results in repairing skin, forming scars that lack appendages such as hair, as opposed to regenerating the skin, forming perfect skin tissue, as seen in fetal wound healing. The absence of hair follicles is thought to promote scar tissue formation during the repair process.

Table 15.1 Phase of wound healing.

Phases	Description
Coagulation	This phase starts immediately following tissue damage. • Injured blood vessels are constricted (vasoconstriction). • Platelets become activated to form fibrin clot (composed of platelets, collagen, thrombin, and fibronectin), which stops blood flow. • Fibrin clot provides scaffold for inflammatory cells e.g., neutrophils, monocytes and macrophages.
Inflammation	This phase starts immediately following tissue damage. • Fibrin clot and damaged tissue release cytokines (TGF-β, PDGF, EGF, FGF, interleukin 8 (IL8/CXCL-8)), which are chemo-attractants to neutrophils that remove bacteria and cell debris. • Monocytes are recruited 24–96 h post tissue damage and transform to activated macrophages, which mediate inflammation, host defense removal of apoptotic cells and promote cell proliferation and tissue restoration. • In the early stages of inflammation, macrophages are microbicidal and proinflammatory (M1) and are important for engulfing and destroying pathogen by phagocytosis. Soon after M1 macrophages switch to an antiinflammatory phenotype and become alternatively activated macrophages (M2) which contribute to new blood vessel formation. • Adaptive immune system consisting of Langerhan cells, dermal dendritic cells, and T cells also become activated to ward off infection and clear cellular debris.
Fibroplasia	This phase starts from day 4–lasts around 2–4 weeks. • Angiogenesis occurs which involves proliferation and migration of endothelial cells, branching and formation of new blood vessels. • Fibroblasts proliferate and invade fibrin clot to form contractile granulation tissue; mature into myofibroblasts and contract wound to enable wound closure; produce and deposit ECM proteins including glycosaminoglycans, type III collagen, which eventually forms a scar. • Reepithelialization occurs with migration and proliferation of wound edge keratinocytes and follicle remnants.
ECM remodeling	This phase starts from a 2–3 weeks post injury to 2 years or more. • Type I collagen replaces type III collagen, facilitated by matrix metalloproteinases produced by fibroblasts, macrophages and endothelial cells to strengthen the scar. • Disorganized collagen becomes lamellar. • Mature scar is formed.

Wounds are clinically divided into **acute wounds** such as **burn wounds**, and **chronic wounds** such as diabetic or venous ulcers. Acute wound healing is timely and orderly, whereas chronic wound healing is delayed.

15.2.1 Burn wounds

Large area skin loss is seen in acute trauma including burn trauma, where **skin engineering** has particular relevance. An estimated 11 million people worldwide sought medical treatment for burns in 2004. Most burns are small, occupying less than 10% of total body surface area, and are caused by flame (43%) and scalds (34%), while about 3.6% are from electrical burns and 3.5% from chemical burns. The Burn Incidence Fact Sheet of the American Burn Association (ABA) states that in 2016, a total of 486,000 people sought medical treatment for burns in the United States. There were 40,000 hospital admissions, which included 30,000 required admissions to burn centers. In total, 3275 patients died from burns and smoke inhalation, and the majority of these deaths (2745) came from residential fires; whereas, in all low- and middle-income countries, the estimated total number of deaths per year is 180,000.² Although the incidence of life-threatening burn injury has fallen and the mortality improved significantly in high-income countries, such burn care remains a significant financial burden. Burn wound depth assessment is vital to wound management. Early wound closure is the key to increasing survival rates and limiting mortality from sepsis and scar morbidity. The first attempt to classify burn depth was made by Jean de Vigo (1460–1525) of Italy in 1483, who recognized superficial and deep wounds, but it was not until 1953 that Jackson led current classifications.³ Jackson's description includes **superficial partial skin loss** (affecting epidermis and **papillary dermis**), **deep partial skin loss** (affecting the epidermis and papillary and **reticular dermis**), and **full-thickness skin loss** (affecting the epidermis, dermis, and subcutaneous tissue). Poor and delayed healing is associated with the latter two, recommending skin grafting in those cases. Since then, the use of **skin substitutes** has also been used as forms of treatment.

Successful treatment of large burn injuries requires early excision of the burn wound followed by closure. Different traditional treatments for burn wound coverage exist. **Autografts** (including both **split-thickness** and **full-thickness grafts**), **allografts**, and **xenografts** can be used for the resurfacing of burn wounds. A split-thickness skin graft is the gold standard treatment as it provides permanent cover. According to the Healthcare Cost and Utilization Project, over 160,000 skin grafts are performed annually in nearly one out of three burn hospitalizations. Their use is popular provided adequate donor tissue is available. However, implementation of split-thickness skin grafts can be limited where the burn injury involves a large total burn surface area, with sparse donor sites and potential for further pain and scar formation. Allografts and xenografts are temporary, subject to rejection, and carry the risk of disease transmission. In recent years, **skin replacement products** (**dermal templates**, **epidermal sheets**, and **full-thickness skin equivalents**) have also been developed to provide permanent wound closure, thus preventing mortality from sepsis and scar morbidity.

15.2.2 Chronic wounds

Chronic wounds are nonhealing wounds that develop due to an interruption in the normal wound healing process resulting in a delay and tissue functionality not being restored within 3 months. Several factors can cause the delay including venous or arterial insufficiency, diabetes, renal disease, local pressure effects, trauma, and advanced age. Local factors such as tissue hypoxia, ischemia, maceration of

tissue, foreign bodies, exudates, infection, dysregulation of the inflammatory process, and systemic factors including compromised immune or nutritional status can all impair healing.⁴

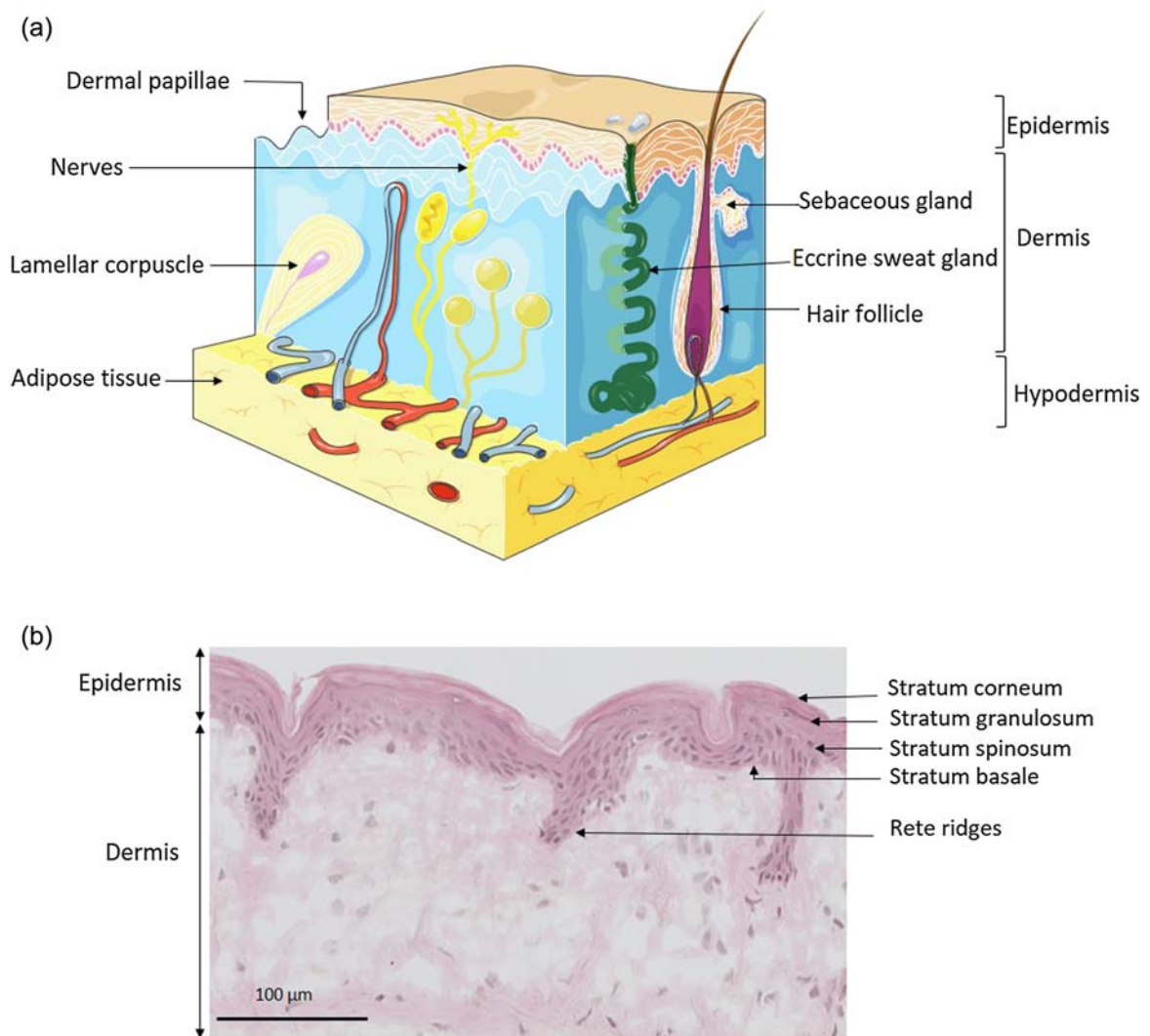
The increased prevalence of noncommunicable diseases such as diabetes, obesity, and vascular disease contributes to the rise of chronic wounds. **Diabetic foot ulcers**, **venous leg ulcers**, and **pressure ulcers** are the most common chronic wounds and generate a major global problem with high management costs. In the United States alone, over 6 million people are afflicted with chronic wounds, and the overall annual medical cost for treatment is around \$25 billion, thus placing a significant burden on the healthcare system.⁴ Clinical treatments for these wounds include operative debridement, flap repair, antibiotic therapy, platelet-rich plasma therapy, frequently changing dressings, hyperbaric oxygen therapy, and negative pressure to improve blood supply.⁴ New forms of treatment have been explored including cell-based therapy, biological dressings, and dermal substitutes, which may offer some benefit. Cultured keratinocyte allografts and autografts have also been used as therapies.

Healthcare systems need alternative treatment approaches to potentially improve wound healing and normal skin tissue architecture. With increasing clinical need for more advanced and efficacious treatments, more innovative solutions are needed to reduce healthcare budgets. Elucidating the underlying molecular and cellular mechanism surrounding wound healing will lead to a better understanding of these processes and enable more effective treatments to be developed that will benefit large burn injuries and chronic wound cases. With recent advances in wound care technologies, the use of skin tissue engineering and **stem cells** are now gaining widespread recognition. In subsequent sections of this chapter, we focus on skin biology, keratinocyte culture, and grafting. Tissue-engineered skin equivalents are also discussed with emphasis being placed on their biological relevance to wound healing. The clinical application of keratinocyte stem cells and the potential use of other adult stem cells in skin tissue engineering are also discussed.

15.3 Structure and function of the epidermis

The epidermis is a multilayered cellular structure that acts as a formidable barrier to protect the body from the external environment. The main cell type, making up 95% of the total, is the **keratinocyte**; however, it is also comprised of pigment producing **melanocytes**, specialist macrophages called **Langerhans cells** that act as immune sentinels and **Merkle cells** that function mainly as touch receptors. Keratinocytes are **terminally differentiating** cells that move progressively from deep to superficial before being shed from the outer surface as a keratin scale. Morphologically, the epidermis is divided (from deep to superficial) into four layers: the **stratum basale**, **stratum spinosum**, **stratum granulosum**, and **stratum corneum** (Fig. 15.1). Collectively, these cells form a barrier preventing internal organ dehydration and protecting from chemical, biological and UV attack. In addition, the epidermis is an endocrine organ, best known for the synthesis of vitamin D, but can also produce steroid hormones, and is the primary site of estrogen synthesis in postmenopausal women.

The stratum basale consists of cuboidal-shaped keratinocytes, usually one cell deep, in a continuous layer and is attached to the basement membrane. The epibasal layer, the stratum spinosum, is distinguished by numerous **desmosomes** interconnecting the cells to produce a stable network. The desmosomes appear to provide not only a means of intercellular adhesion, but also to form a focus for the internal organization of cytoskeletal components, particularly tonofilaments. The next layer, the stratum granulosum, is characterized by the intracellular deposition of keratohyalin granules, particles

**FIGURE 15.1**

(a) Schematic of normal skin structure, components, and layers. (b) Histological image of dermis/epidermis. *Adapted from Servier Medical Art, Creative Commons Attribution 3.0 France.*

of irregular shape and approximately 2 nm in diameter, which are arranged in rows or lattices. Another feature of this layer is the Odland body—a lamellated granule of 100–300 nm in size containing lipid for discharge into the extracellular space. It is this lipid that binds cells in the stratum corneum. The cells in this layer (corneocytes) become flattened as they lose their nuclei and organelles and as the keratin filaments within them polymerize with the formation of disulfide bonds. This occurs under the influence of filaggrin, the protein component of the keratohyalin granule, and results in the formation of a

flattened, highly insoluble keratin shell bound to the cell membrane by involucrin. Collectively, these processes are termed **terminal differentiation**, forming the cornified barrier that is exposed to the outer environment. Degradation of the intercellular lipid component and the desmosomes precedes the loss of groups of cells as a flake of skin.

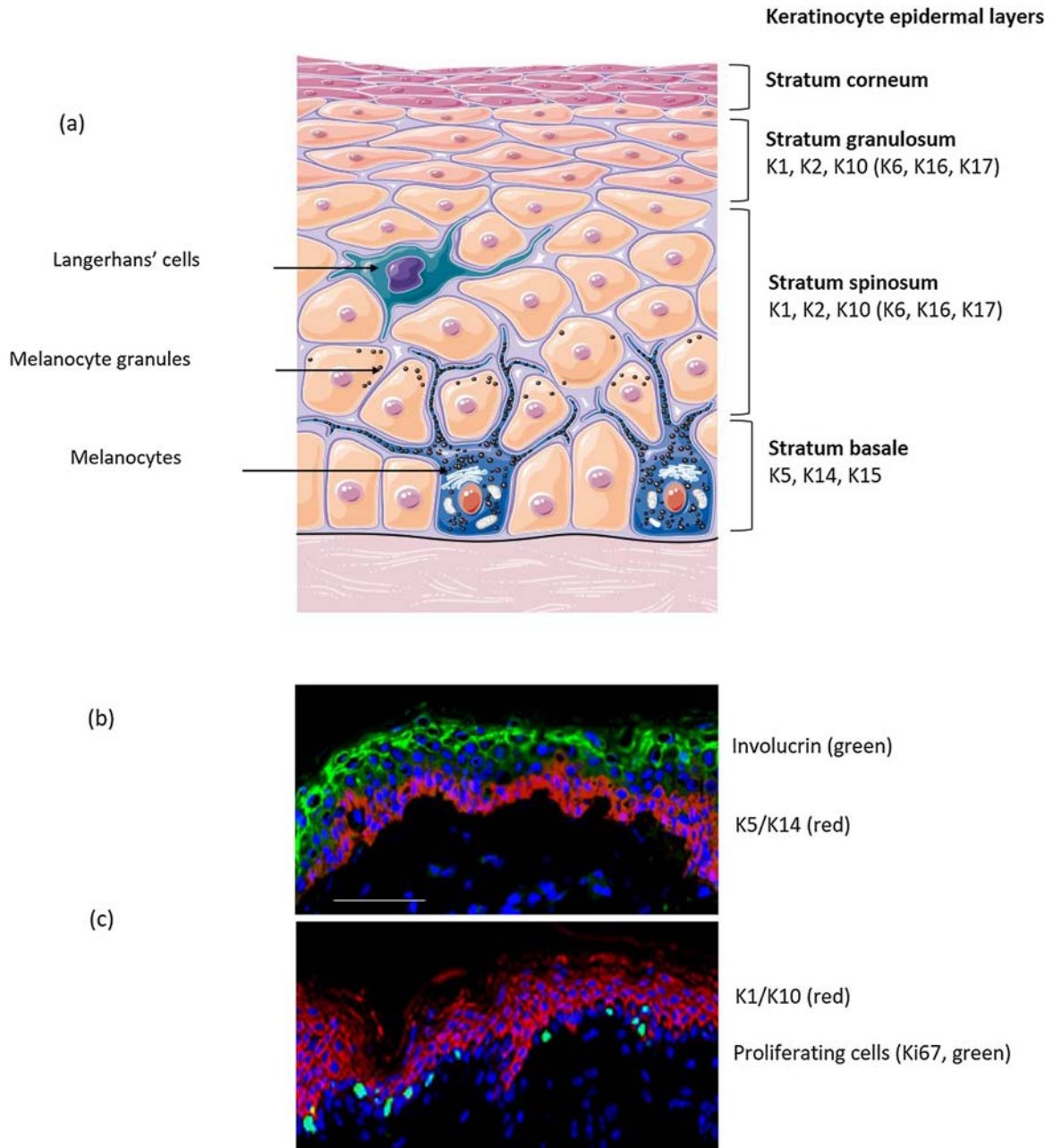
The balance between keratinocyte cell proliferation and differentiation is critical to maintaining healthy skin homeostasis. In the epidermis, replenishment of sloughed off terminal keratinocytes originates from discrete stem cell niches in the stratum basale. There are currently at least two theories, both supported by stem cell fate analysis, for how adult stem cells give rise to terminal differentiating keratinocytes—the classical hierarchical model and the stochastic model of homeostasis.

The kinetics of epidermal proliferation is complicated by the observation that postmitotic cells may be very active metabolically and may increase tissue mass without an increase in cell numbers. The turnover time is the time taken for the whole cell population to replace itself and depends on the length of the cell cycle (the time between successive mitoses) and the growth fraction, i.e., the proportion of cells undergoing mitosis. In normal human skin, the net effect of both parameters is to generate an epidermal turnover time of 52–75 days.

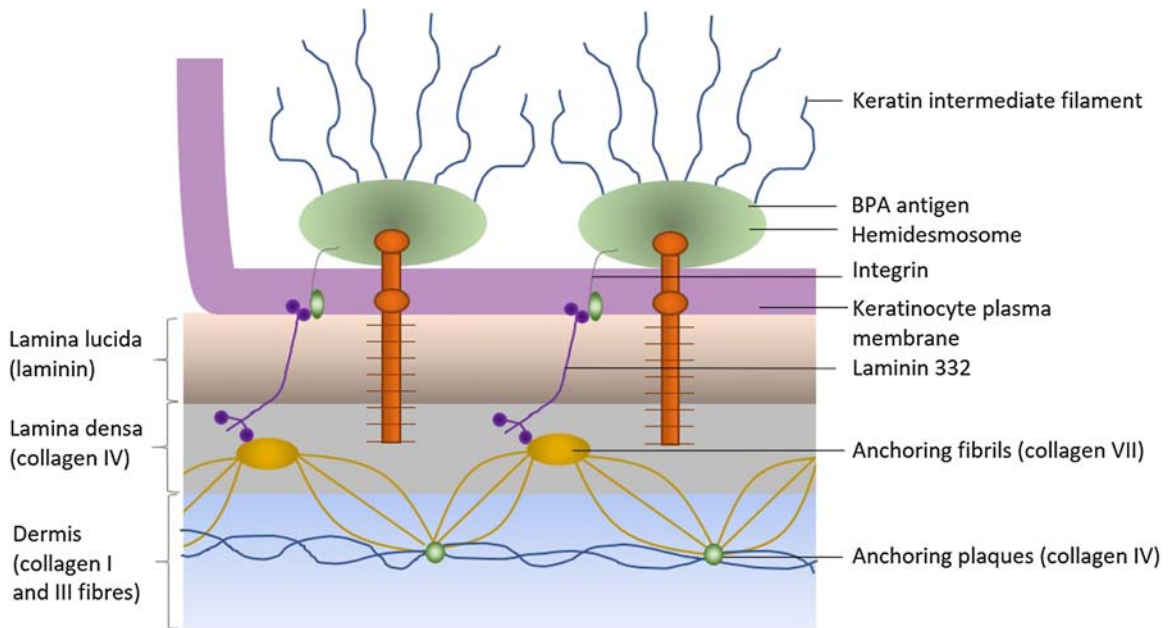
15.3.1 Keratins

The **cytoskeleton** of the keratinocyte is formed from three groups of filaments: actin (7 nm diameter), tubulin (20–25 nm), and intermediate filaments (7–10 nm). The latter group contains several recognized components, including vimentin, desmin, the nuclear laminins, and the **keratins**, the polypeptide molecules characteristic of all epithelial cells and which account for 65% of the final mass of a corneocyte. The keratin family consists of over 30 individual members and are separated by electrophoresis into basic keratins (numbered 1–8) and the acidic keratins (numbered 9–12), and whose corresponding gene families are termed, respectively, types I and II. The keratins pair into acidic/basic dimers that are specifically expressed according to cell physiology and morphology. For example, simple epithelia are characterized by the keratin pair K8/K18 and stratified epithelia by K5/K14. Further, sole and palm skin express K9, differentiated skin K1/K10, and hyperproliferative or regenerating epidermis K6/K16/K17. This expression provides a convenient way with which to investigate morphology and function in the epidermis with the use of immunohistochemistry (Fig. 15.2).

The basal surface of the keratinocyte attaches to the lamina lucida via **hemidesmosomes**. These consist primarily of inner plaques on the inner surface of the cell membrane (allowing continuity with tonofilaments within the cell), outer plaques (closely associated with the cell membrane itself), and the sub-basal plate, external to the cell and immediately adjacent (and embedded into) to the basement membrane. The basement membrane itself may be resolved into three laminae by electron microscopy: the outer **lucida**, **intermediate densa**, and **deep fibroreticularis**. Within the lamina lucida are numerous anchoring filaments related to the hemidesmosomes and orientated perpendicular to the cell and basement membranes. The lamina densa appears relatively amorphous, and the fibroreticular layer appears to demonstrate anchoring fibrils, short, curving structures that extend into the dermis either singly or in relation to amorphous bodies and then termed anchoring plaques. In addition to the above electron microscopic description, several specific molecular components can be identified within the basement membrane; these allow assessment of maturity by specific immunohistochemical staining (Fig. 15.3).

**FIGURE 15.2**

Schematic arrangement of keratins (a) and cell proliferation markers in the different epidermal layers (b and c). *From Servier Medical Art, Creative Commons Attribution 3.0 France.*

**FIGURE 15.3**

The dermoepidermal junction.

The basement membrane is intrinsic to the stability of healing wounds.⁵ Delineated the pattern of basement membrane component deposition with healing over a period of 10 days and showed a near-linear production of most components. Initially, **collagen VII** is absent, while **bullous pemphigoid antigen (BPA)** and **collagen IV** are the predominant components. Collagen VII production only begins after day 3, by which time BPA, **laminin**, **fibronectin**, and **collagen IV** are present in appreciable quantities. By day 7, the levels of all these components are relatively equal and the basement membrane may be described as fully formed.⁵

15.4 Structure and function of the dermis

The dermis is a complex and dynamic matrix of fibrous connective tissue between the epidermis and hypodermis. It provides the skin with structural support, tensile strength, and elasticity. Collagen is the key structural component forming 70% of the dry weight of the dermal matrix. Of the 20 types of collagens, **type I** and **type III collagen** are the most abundant in the **dermal matrix** and type IV and VII reside predominantly at the DEJ. There are several skin appendages and structures supported in the dermis. Buried deep in the reticular layer, the **lamellar (Pacinian) corpuscles** form mechanoreceptors sensitive to pressure, and **Ruffini corpuscles** sense skin stretching. Hair follicles, sweat glands, sebaceous glands, and nerves span the dermis and the epidermis. There is considerable cross talk between the dermis and the epidermis. **Dermal papillae** extend up into the epidermis (Fig. 15.1), increasing surface area contact with the epidermis, where the vasculature from the dermis nourishes

the avascular epidermis and are important for thermoregulation. Environmental cues or epidermal damage can alter dermal matrix deposition, forming a critical response in wound healing. Dermal **fibroblasts** are the primary cells within the dermis, responsible for producing connective tissue (elastic fibers and ground substance composed of **glycosaminoglycans**, which produce **proteoglycans**) and collagen. There are distinct subpopulations of adult dermal fibroblasts rising from at least two different lineages, with differing functions.⁶ Fibroblasts arising from the upper epidermis regulate hair growth and function, whereas fibroblasts arising from the lower dermis (reticular fibroblasts) are responsible for collagen formation and matrix deposition that is particularly important in wound healing. This difference in fibroblast **niche** and function is partly attributed to the induction of scar formation and absence of hair follicle regeneration during the wound healing process in humans.⁶

15.5 Epidermal and hair follicle stem cells of the skin

Skin has the natural ability to regenerate in part from undifferentiated stem cells that are capable of dividing and replicating throughout life. These stem cells can differentiate into multiple cell types with specialized functions and reside in locations called niches (see Chapter 2 Stem cells). In the interfollicular epidermis (IFE), stem cells are found in patches or clusters related to the DEJ along the basal lamina. There is probably a segmental distribution of progeny related to a stem cell, resulting in their patchy distribution along the DEJ. In glabrous (hairless) skin of the soles and palms, the clusters are found deeper, at the tips of the deep *r  te* ridges. Estimates vary as to how abundant they are, but probably 5%–10% of the basal population is stem cells. The basal stem cell population of the IFE is characterized with having high expression of certain markers (Table 15.2). The “bulge region” of the hair follicle, a morphologically distinct region in mice but not humans, has been identified as a major reservoir for stem cells in hair-bearing skin. Recently, however, multiple new hair follicle stem cell

Table 15.2 Some examples of epidermal and hair follicle stem cells and their niches and markers.

Stem cells	Niches	Markers
Interfollicular epidermal stem cells	Basal layer	p63, β 1 integrins, α 6 integrin, Endothelial protein C receptor, Lrig1+, Melanoma-associated, chondroitin sulfate proteoglycan, CD117
Hair follicle epithelial stem cells	Bulge region	K15, CD34, Lgr5, Sox9, Lhx2, NFATC1, CD200, K19
	Upper isthmus/junctional zone region	Lrig1, MST24, ABCG2 transporter
	Lower isthmus	Lrg6, Gli1
Hair follicle dermal stem cells	Hair germ at base of hair follicle	K15, Lgr5, Gli1
	Dermal papilla (DP) in the hair bulb	CD90, CD133, Sox2
	Dermal sheath (DS) contiguous with DP and surrounding hair follicle	CD105, CD29, CD49b, CD49d, CD73, CD271, GD2
Sebaceous gland stem cells	Sebaceous glands, infundibulum	Blimp1
Melanocyte stem cells	Hair follicle bulge region and hair germ	Dct, Sox, Pax3
Neural progenitor cells	Bulge region	Nestin

populations have also been identified outside this anatomical area that have differing abilities to contribute not only to the hair follicle itself but also to the IFE and the sebaceous gland (Table 15.2). Under normal conditions in healthy and undamaged skin, these heterogeneous pools of stem cells in the IFE and hair follicle stay restricted within their respective niches in the skin. However, following tissue injury, IFE and hair follicle stem cells are able to contribute toward different lineages during wound healing and reepithelialization. One study in mice demonstrated that stem cells from hair follicles at the wound edge migrated to the center of the wound, leading to full hair follicle regeneration when a large wound was left open to heal.⁷ This work showed the importance of the hair follicle for full fidelity skin repair. It is not yet known whether the same phenomena are observed in humans, since the risk of infection makes leaving wounds open impractical. Other animal wound model studies have shown that hair follicle stem cells applied to wounds directly or via skin scaffolds promote wound healing by accelerating wound closure and reepithelialization, while increasing collagen deposition, tensile strength, and angiogenesis. Furthermore, hair follicle dermal cells can generate hair bud-like structures. The properties of epidermal and hair follicle stem cells make them promising candidates for wound healing and tissue engineered products.

15.5.1 Keratinocyte stem cells

The keratinocyte stem cell (KSC) (holoclone) has extensive proliferative capacity but cycles slowly, minimizing potential DNA mutations, and probably consequently is long-lived. The transcription factor p63, a p53 homologue required for epithelial development, has been shown to be “abundantly expressed by epidermal and limbal (cornea) holoclones,” *in vitro*. Each holoclone stem cell can divide many times (more than 140) into another copy and a separate daughter cell. The resulting transient amplifying (TA) cell (meroclone) may divide a number of times providing an explosion in cell numbers, vital in a high turnover environment, before further differentiation into a terminal cell with limited proliferative potential (paraclone), which migrates away from the basal layer. Various models have been proposed on how stem cells contribute to maintaining epidermal homeostasis. The classical hierarchical model proposes the presence of epidermal proliferative units (EPUs), with stem cells at the EPU center and TA daughter cells at the EPU periphery. These stem cells divide a finite number of times in the basal layer and produce TA cells that then progress to different cell lineages until ultimately undergoing terminal differentiation. Recently, however, other stochastic models have challenged the original model. One stochastic model proposes that stem cells can divide an indefinite number of times into two terminally differentiated cells, two undifferentiated basal cells or one of each. Another model proposes two types of epidermal stem cell populations that are spatially distinct or present in different locations of the basal epidermis but can contribute to each other’s territories upon wounding. Reports from mice studies support the existence of these models and may be attributed to epidermal variation across the different anatomical skin sites.

15.6 In vitro keratinocyte culture

Advances in *in vitro* **keratinocyte culture** have been driven by a clinical need to replenish the epidermis and provide wound closure (e.g., due to burn injury, chronic wound, or skin ulceration). Human skin is a readily accessible organ, often discarded during routine plastic surgery and biopsied with relative ease.

As a result, many breakthrough discoveries in stem cell research and tissue engineering have been achieved with skin cells as the initial source of biological material. Furthermore, an outstanding study recently combined skin stem cells in a skin culture model with **gene therapy** and provided an entire replacement of the epidermis in a patient with the blistering and life-threatening disorder, junctional epidermal bullosa⁸ as outlined in the classical experiment text box.

In the mid-1970s, Rheinwald and Green published their chance discovery that human epidermal cells proliferated under the same conditions as mouse teratoma cells. Significantly, it was the provision of a feeder layer of lethally irradiated Swiss 3T3 mouse fibroblasts and of mitogens within the culture media that enabled human epithelial cells to proliferate rapidly without contamination of human dermal fibroblasts. This work proved one of the most important breakthroughs in the field since it enabled the expansion of human keratinocytes on a **monolayer** in a tissue culture flask (Fig. 15.4). Panel (a) shows advances in Keratinocyte's culture, enabling applications in patient care for burns and skin ulceration, skin testing for cosmetics and consumer goods as well as advanced testing for drug development. Panel (b) highlights morphological differences in keratinocytes cultured at low and high calcium. Further work⁹ optimized the growth conditions resulting in a mean cell cycle time of 22–24 h, much faster than that seen in either normal or hyperproliferative (psoriatic) skin.

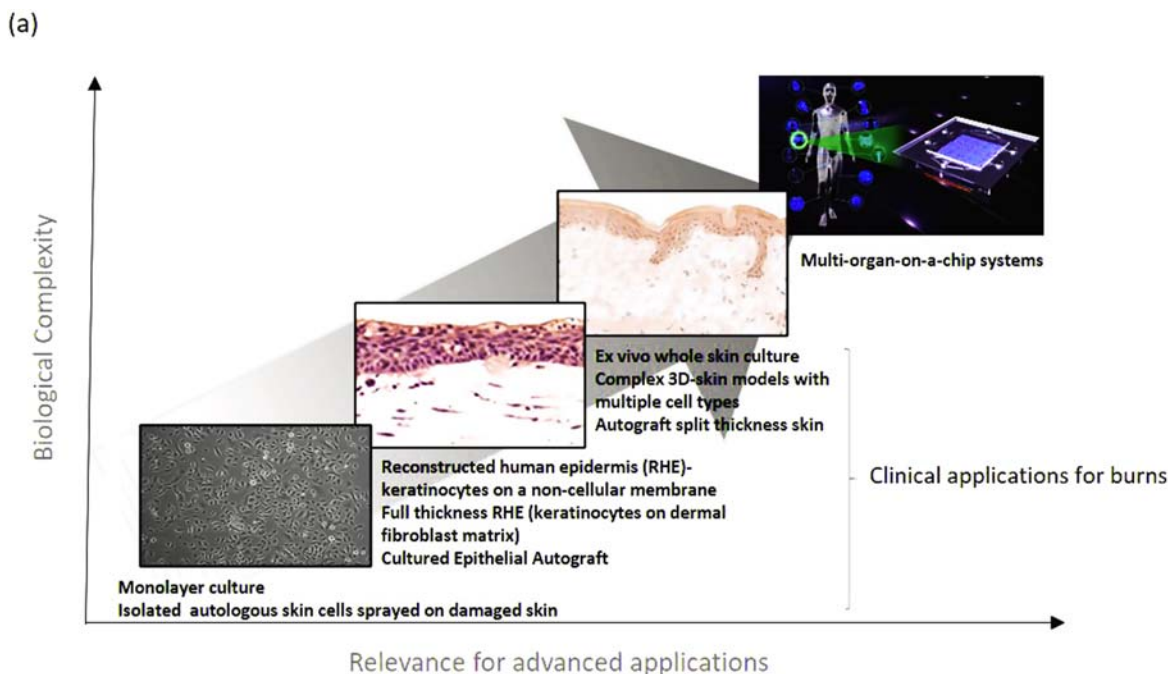
Further modifications to keratinocyte culture medium are continually progressing. Keratinocytes can also be expanded in low calcium, feeder-free, and serum-free conditions (xenobiotic-free culture), and more recently, animal-product free culture media (used for the testing of vegan-friendly cosmetics), which is favorable in removing contaminating cells such as fibroblasts, but their proliferative life span is reduced. In optimal growth conditions, keratinocytes isolated from a 3 cm² piece of skin can be expanded more than 5000-fold within 3–4 weeks, yielding sufficient sheets of keratinocytes to cover the entire surface of an adult human.

There are many factors that affect keratinocyte culture, the most important being the age of the donor. Population doublings of cells tend to decrease with age. Moreover, both fibroblasts and keratinocytes have a finite number of population doublings, termed the **Hayflick limit**, after which they are unsuitable for culture. Systemic illness in donors may decrease the proliferative capacity of keratinocytes. In addition, the composition of the clonal types, i.e., holoclones, meroclones, or paraclones generated from the keratinocyte culture, is important.

Within the culture flask, proliferating cells form islands, which spread laterally and, to a lesser extent, stratify. Growth is predominantly as a monolayer in a horizontal plane, but the daughter cells of those in the center of the island start to stratify. If the entire flask is allowed to stratify, a so-called “**Green sheet**” is formed, consisting of up to 12 cell layers. The number of layers is determined by the calcium ion concentration in the medium; low calcium solutions promote the formation of a monolayer, and high calcium solutions that of a stratified sheet.

15.6.1 Cultured keratinocyte sheet grafts for the treatment of burns

Such rapid keratinocyte growth allowed for its utilization in the clinical management of expansive burn injury, as opposed to split-thickness skin grafts. A split-thickness skin graft involves the removal of a wafer-thin piece of skin including the epidermis and part of the dermis, using a dermatome from a



(b)

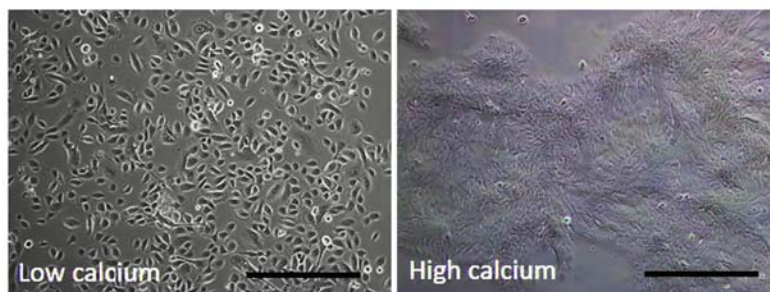
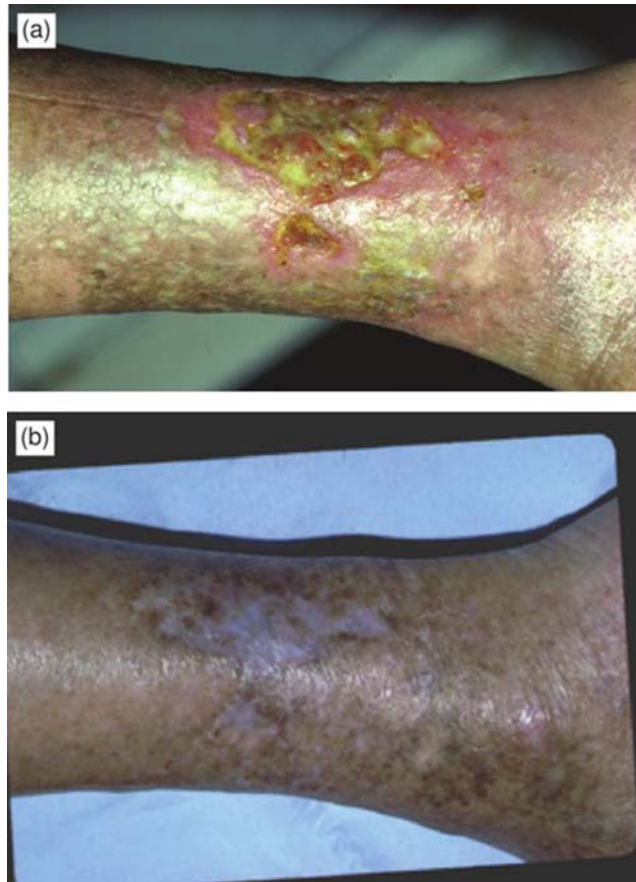


FIGURE 15.4

Advances in keratinocytes culture, enabling applications in patient care. Scale bar = 200 μm .

healthy site, and grafting the skin directly on to the damaged region. In some cases, this is either inappropriate or not possible due to the extent of the damage and inflicts further injury to a patient who may already be critically ill. Thus, advances in keratinocyte tissue engineering enabled the development of autologous (from the patient) engineered sheets of keratinocytes, transported via carrier dressings or cultured on a dermal matrix (Fig. 15.5) to be grafted back onto the patient wounds. The keratinocyte sheet, also known as **cultured epithelial autograft (CEA)**, is used in the treatment of partial- and full-

**FIGURE 15.5**

Cultured keratinocyte grafts used to cover a leg ulcer before (a) and after 3 weeks (b).

thickness burns and venous and diabetic ulcers. Cultured keratinocytes have also been used to treat a range of diseases at a variety of anatomical sites (Table 15.3).

Histologically, cultured keratinocyte sheet grafts consist of sheets of cells with much simpler organization than that of native epidermis. The basal layer is relatively well formed, but the suprabasal layers are irregular in thickness, consisting of 2–10 layers, with poor organization. Although desmosomes and gap junctions are present, clinically the skin is extremely fragile on full-thickness wounds due to a flat dermoepidermal junction. There are also challenges with culturing skin cells from patients within a suitable timeframe in a **good clinical practice**-compliant environment (see Chapter 22 Clinical Translation) such that alternative methodology is more practical. One option to avoid long waiting times is the use of cultured epithelial allograft (from a nongenetically identical donor). However, this is used as a temporary covering as allografts do not persist since they are subject to **immunological rejection**. Alternative cell technology for burn and wound healing includes harvesting autologous skin cells

Table 15.3 Applications of cultured keratinocyte technology.

Autologous	Allogeneic
Intraoral	Chronic venous ulcers
Mastoid cavities	Diabetic leg ulcers
Superficial burn	Skin graft donor sites
Full-thickness burn injury	Burn injuries
Facial scar revision	
Chronic ulcers	
Giant congenital nevi	
Hypospadias	
Separation of Siamese twins	
Recessive epidermolysis bullosa	

with using the commercially available ReCell kit from Avita Medical. This technology sprays a noncultured mixed cell population consisting of keratinocytes, fibroblasts, and melanocytes directly to the wound to enable reepithelialization. However, a drawback of this technology is that ReCell lacks a dermal matrix, and it is unclear how many cells “take” to the skin.

15.7 Cultured three-dimensional skin models

Keratinocyte culture progressed from monolayer methodology to three-dimensional (3D) skin modeling in 1981, where expanded keratinocytes were seeded on **deepidermalized dermis** (cadaver skin with the epidermis and all cellular components chemically removed) that generated some form of keratinocyte stratification. Advances in creating a dermal matrix led to the first patent of a “Living Skin Equivalent” in 1984 forming a culture of keratinocytes on top of a collagen matrix containing dermal fibroblasts.¹⁰ This formed the basis of the first commercial living skin equivalent model in 1991. These models were cultured in a tissue culture incubator, initially with all cells submerged in culture media. After approximately 7 days, the cultures are raised so that the keratinocytes are exposed to an “air–liquid” interface (ALI) to promote stratification. Lifting to the ALI is a key process in all 3D keratinocyte culture.

Fortunately, 3D skin engineering is advancing at a pace, due to the need for accurate human equivalent skin models to be used in clinical applications and personalized medicine as well as for cosmetic testing, pharmaceutical testing, and drug development (Fig. 15.4). Traditional animal models (the Draize test) only offer 59% accuracy of predicting human skin irritation and are poor models of the human skin barrier, highlighting the requirement for more suitable test methodologies.¹¹ Moreover, a number of directives to reduce animal testing, culminating in the 2013 EU animal testing ban for cosmetics, competitively advanced skin tissue engineering for skin irritation and sensitization testing. There are now at least 18 companies offering 3D human equivalent skin models for this purpose. Technology for 3D skin models is evolving from the simple keratinocytes stacked on a matrix (reconstructed human epidermis) and dermal–collagen matrix with keratinocytes stacked on top (termed full-

thickness reconstructed human epidermis), to become more representative of complex multicellular skin physiology.¹²

There are four key areas undergoing intensive research and development, namely engineering with fluidics, biological vasculature formation, incorporation of multicellular components and generation of disease models. Cell culture was formally conducted using well plates and tissue culture flasks, the media is static and needs to be manually changed every 2–3 days to ensure fresh nutrient supply. Advances in engineering are enabling the development of fluidic technology, demonstrating cells and tissue display improved human-equivalent physiological parameters in culture with continuous media perfusion. These systems have evolved from simple millifluidic scale, to microfluidics, with new advanced organ-on-chip technology where representations of different organs are incorporated in one flow model to simulate whole-human body systems (see Chapter 20 Organs on a chip).¹³

New engineering techniques in **3D printing** (see Chapter 10 Microfabrication Technology in Tissue Engineering) and materials science are enabling modeling of vascular perfusion in gel-based dermal matrices. These systems are working to enable extensive nutrient delivery throughout 3D models akin to the capillary network. Early 3D skin models simply incorporated keratinocytes and dermal fibroblasts. While these are the predominant cell type in the epidermis and dermis, respectively, these systems are not representative of the full skin system. Classically, cell culture has been developed in a reductionist approach with culture media developed to support an individual cell type. Critical culture chemistry can differ between cell type, such that incorporating and maintaining multiple cell types in one system are not trivial. Some systems are attempting integration of **induced pluripotent stem cells (iPSCs)** with chemical stimulation to create multiple-cell type 3D skin constructions.¹³ Of particular interest is the incorporation of an immune response by including resident immune cells (such as Langerhans cells) to aid development of representative inflammatory skin responses. Other advances include the incorporation of hair follicles, although currently this is a low-throughput process and may not include the sebaceous gland. Hair follicles are one of a primary route of transdermal drug delivery, creating pockets that capture topical drug in a region with a less developed cornification to facilitate absorption.¹² The skin microbiome is also garnering much attention, differences in commensal bacteria and onset of infection are associated with a broad spectrum of skin conditions from poor wound healing, chronic wounds, atopic dermatitis, and dandruff. New coculture skin models with bacteria have been reported for in vitro chronic wound models and cosmetic testing.

An advantage of 3D skin models over ex vivo culture (culturing excised whole skin tissue) is the creation of disease models. Although healthy skin is relatively abundantly available through plastic surgery, diseased skin is harder to obtain, requiring invasive clinical access to small (2–6 mm) skin punch biopsies. Approaches to creating disease models such as psoriasis and atopic dermatitis include applications of chemical and cytokine cocktails to healthy skin and healthy 3D skin models; isolation and expansion of keratinocytes with dermal fibroblasts from patients with skin conditions; use of iPSCs to model disease states, engineering to integrate cellular defects in cultured keratinocytes and/or dermal fibroblasts for 3D skin models to mimic a skin disease.

Despite the above, fundamentally there are still essential improvements required to create competent cornification of the epidermis. Currently, 3D skin models are typically 5–50-fold more permeable than human skin, and there is no system capable of replicating the human skin barrier. Some reports suggest

that this is due to the lack of integration of hair follicles including the sebaceous gland that offers natural moisturizing factor to the epidermis; however, it is more likely due to the inherent changes that the mere act of culture induces. Transcriptomic analysis demonstrates keratinocytes rapidly adopt a semi-wounded, partial diseased skin state once the skin is excised from the body, which persists with all forms of keratinocyte culture. While traditional culture media has been designed to induce rapid cell expansion to provide an abundance of biological material, it has done so at the expense of maintaining the “healthy” cell type. Thus, it is not surprising that healthy keratinocytes adopt a phenotype partially akin to psoriasis or a wound-healing response. Identifying methodology to address this problem is critical in advancing all forms of skin tissue engineering.

15.8 Immunogenicity with allogeneic and biosynthetic materials

It has been established that allografted keratinocytes do not persist in an immunocompetent host¹⁴; however, they have been used to accelerate partial thickness wound healing by providing growth factors. **Langerhans cells** are epidermal dendritic, antigen-presenting cells that initiate the immune response, including the recruitment of CD4+ and CD8+ **T cells**, leading to allograft donor rejection. It is possible to deplete Langerhans cell number by in vitro UV irradiation, and this may attenuate the rejection response to allograft.¹⁵ Langerhans cells proliferate poorly if at all in vitro. Studies have demonstrated prolonged but not indefinite survival of grafts with Langerhans cell depletion while UV irradiation postgrafting appears to prolong allograft survival.¹⁵ The latter cannot be repeated indefinitely, however, and would be expected to adversely affect wound healing. Recruitment of T cells, driven by a mismatch in donor major histocompatibility complex (MHC) and recipient T cells, triggers immune cell infiltration causing **allogeneic** skin graft rejection.¹⁶ Systemic approaches to prevent allograft loss are the same as standard protocols adopted to prevent organ transplant rejection, including Cyclosporin A administration, alone or in conjunction with antibody administration, or as a combination of UV irradiation and 8-methoxypsoralen.¹⁵ Newer gene therapy approaches include activating the IL-10 cytokine response and suppressing the IL-1/IFN-gamma Th-1 immune response to suppress T cell recruitment.¹⁶ Ultimately, however, there appears little doubt that transplanted allogeneic keratinocytes are lost from the wound.¹⁴

Biosynthetic materials can also initiate inflammatory responses termed “**foreign body response**” through the attraction, adhesion, and activation of phagocytes to the implant site. In some cases, this can be used to activate the repair process by promoting migration and proliferating of dermal fibroblasts.¹⁶ Thus, the choice of auto- versus allogeneic skin graft or biomaterial will determine whether the repair is temporary, short-term requiring further intervention or a permanent covering. Regardless, early excision of damaged skin tissue with grafting in the first 3 days leads to an increased survival rate and reduces mortality.

15.9 Development of in vivo somatic keratinocyte stem cell grafting

15.9.1 Evolution of epidermal replacement

Early keratinocyte culture developed from the 1930s onward. However, cultured keratinocyte characterization was not performed until 1956. The fact that epidermal cells were able to proliferate in culture

was clear by 1960. Early keratinocyte differentiation occurred in the absence of fibroblasts. The classic Rheinwald–Green technique was not published until the mid-1970s.⁹

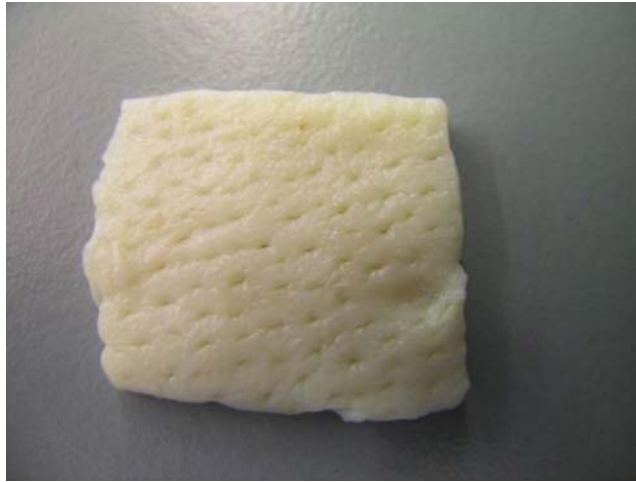
Although keratinocyte expansion in vitro has many potential clinical applications, keratinocyte attachment, proliferation, and differentiation depend on interaction with the dermis. Key to the development of stable epidermal replacement has been the study of the interactions between the epithelial and mesenchymal layers. Initial excitement was dampened by early reports of poor “take,” fragile epidermis, and late blistering. Autologous grafting became a clinical reality in 1981 with the grafting of two patients suffering 40% and 80% total body surface area (TBSA) burns.¹⁷ Only a small portion of their total wound areas was grafted, but it appeared that graft “take” was superior on newly excised and fresh wounds rather than granulating wounds. Reported successful engraftment has been highly variable, between 0% and 95% mean “take.” Even experienced teams have been unable to attain the same engraftment rates as with split-thickness autografting. A case series of 88 burns with a mean graft “take” of 73% with a 91% overall patient survival rate has been reported.¹⁸ In the same study, some of the complications included early blistering and shearing, pruritus and itching, late CEA loss, and wound contractures.¹⁸

15.9.2 Evolution of dermal replacement

A dermis or equivalent is essential to the “take” and quality of cultured keratinocyte grafts as dermoepidermal interaction and signaling play a key role in skin homeostasis and wound repair. Functional and cosmetic outcomes are superior when more dermis is included in split-thickness or full-thickness skin grafts as these limits scarring and **wound contracture**. In massive burn injury, there is a limited donor area for split-thickness skin graft harvest, and whole allograft is transplanted to provide temporary wound closure. Allograft aids in the preparation of the wound bed, reduces pain and infection rates, as well as decreases protein, electrolyte, and fluid loss. It is the epidermal component of the allograft skin that primarily results in rejection. In large burn injuries there is a suppression of **adaptive immunity** (immediate first line of defense against pathogens, e.g., through skin injury mediated through phagocytosis by neutrophils and macrophages etc.), **innate immunity** (slower, second line of defense against pathogens performed by lymphocytes), and initial immunological and clinical “take” with vascularization of the graft over some weeks. Removal of the alloepidermis after engraftment and its replacement with cultured autologous keratinocyte sheets was a technique developed to limit rejection. The presence of the allodermis also stimulates epithelialization. Other forms of dermal replacement have been developed; an early example comprised fibroblasts incorporated into a glycosaminoglycan membrane.

15.10 Poor keratinocyte “take”

The reasons for cultured keratinocyte sheet graft failure are complex, but possibly the most important factor is the state of the wound bed engrafted. Both the quality of the tissue and the bacterial load are significant factors that adversely affect cultured keratinocyte take. Therefore, early excision of the burn wound reduces bacterial colonization and enhances keratinocyte take. Paradoxically, wound bed preparation with topical antiseptics can damage keratinocytes. Cultured keratinocyte grafts are fragile on

**FIGURE 15.6**

Glycerol-preserved cadaveric skin used for preparing DED. A 1.5×1.5 cm square of DED is shown.

full-thickness wounds due to a flat dermoepidermal junction and are prone to blistering, thus influencing take rates. However, take rates can be improved by the use of dermal equivalents.

One of the earliest dermal equivalents, deepidermalized dermis (DED), has been used to support keratinocyte growth and treat full-thickness burn wounds (Fig. 15.6). It is derived from cadaveric skin, which is washed with phosphate-buffered saline containing antibiotics and left in the solution at 37°C for up to 5 days. The incubation period removes all cellular components; the epidermis is then mechanically removed using forceps leaving behind the acellular DED. Other bioengineered dermis have also been created based upon a simple molecular scaffold.

15.11 Skin tissue engineering

Early studies in the late 1970s laid the groundwork for the development of engineered human **skin equivalents**, which are some of the most well-established and effective tissue engineering products on the market. Like many engineered tissues, skin equivalents employ various combinations of living cells, biomaterial scaffolds, and in vitro cultivation to promote the regeneration of normal, healthy skin. To date, there have been a range of approaches including dermal templates, epidermal sheets, and full-thickness skin equivalents consisting of both dermal and epidermal components. **Biomaterial scaffolds** may be synthetic or natural, autologous or allogeneic, and fabricated by various techniques such as electrospinning, phase separation, freeze-drying, self-assembly, and 3D printing. These scaffolds may or may not include cells and may vary in cost, engraftment, risk of disease transmission, handling, and storage (see Tables 15.4 and 15.5). This section will highlight specific examples of engineered skin technologies and their therapeutic and clinical applications and is divided up into subcategories based on the foundations of their technology including acellular dermal matrix, dermal matrix with

Table 15.4 Examples of replacement matrices.

Dermal replacements	Epidermal and dermal combination replacements
Cryopreserved allograft	Apligraf
Glycerol preserved allograft	Orcel
Alloderm	DenovoSkin
Integra	
Dermagraft	
Matriderm	

fibroblasts, dermal matrix with keratinocytes, skin cell suspensions (applied as sprays or injections), and full-thickness skin equivalents.

15.11.1 Acellular dermal matrix

Integra dermal regeneration template

Dermal regeneration templates were some of the earliest biomaterials to be translated to clinical practice. The initial collagen lattice developed by Bell was modified to include chondroitin-6-sulfate for biochemical stability and became marketed commercially as **Integra** and has been approved by the FDA (Fig. 15.7). It was derived from bovine collagen and shark chondroitin sulfate and covered by a silicone surface layer to aid delivery. Integra allows immediate coverage of large, acutely injured wounds, reducing bacterial ingress and water egress.^{21,22} It has also been used successfully in the treatment of chronic wounds. The porous scaffold structure (70–200 μm) allows migration of endogenous fibroblasts and endothelial cells into the dermal matrix producing a vascularized neodermis. In the original system, there remained a delay of 3 weeks before the definitive epidermal cover was formed. Integra can also be used as a bed for sheet split-thickness skin grafts and at best provides the quality of a full-thickness skin graft from a split-thickness skin graft donor site. Another approach that has evolved combines widely meshed split-thickness autograft, where the graft is expanded by cutting holes in it, with cultured autologous keratinocytes, fibroblasts, or noncultured cell suspension (ReCell—see below) as adjuvants. Other dermal templates are now also available, including Alloderm and Matriderm.

AlloDerm

AlloDerm is an acellular human allogeneic dermal matrix preserved by freeze-drying. The epidermal basement membrane is also preserved, and it can be used to prepare a full-thickness wound bed before application of thin split-skin graft following burn excision. More recently, AlloDerm has been used in other applications including alloplastic breast reconstruction, abdominal wall reconstruction, **rhinoplasty** or nose job, vaginal repair, and radial forearm free-flap donor site coverage. Although this product has found its marketplace as a subcutaneous filler in aesthetic procedures, other similar decellularized products are increasingly becoming available from tissue banks, e.g., Glyaderm from the Euro Tissue Bank and from UK National Health Service Blood and Transplantation (NHSBT).

Table 15.5 Current tissue engineering methodologies for managing wounds and burns.

Technology approach	Trade name/ Proper name	Manufacturer	Indications	Approved by/date	Dosage	Description	At/ AL	Price (US \$)	Usage
Bilayered sheets	Apligraf	Organogenesis Inc. and Novartis AG (United States)	Chronic VLU, DFU	US FDA 2000 June	Circular disk with diameter and thickness of 75 mm and 0.75 mm in size	Bilayered bioengineered skin with inner layer of neonatal HDF and outer layer of neonatal HEK	AL	\$1500 –2500 per treatment	1million units in 2017
Bilayered sheets	JACE Epidermis-derived cell sheet	J-TEC (Japan)	Scars, vitiligo, nevi (birthmarks), ulcers, skin-graft donor sites, severe burns: DDB + DB $\geq$ 30%	Japan PMDA 2007 October	Sheet consisting of 1×10^4 cells/cm ²	Keratinocyte sheet cultured on 3T3-J2 cells	AT	\$3140 per 8 × 10 inch sheet	Not published, but predicted to generate \$100m-\$300m in revenue in Japan in 2021
Bilayered sheets	Bilayer artificial skin	Shaanxi Eyre skin Biological Engineering (China)	Deep partial-thickness wound, not more than full-thickness burn wound 20 cm ² (diameter < 5 cm)	China CFDA 2007	NA	Bilayered artificial skin with epidermal layer composed of human epidermal cells and dermal fibroblasts from human and bovine collagen	AL	NA	
Bilayered sheets	DenovoSkin	Cutiss AG (Switzerland)	Treatment of adult and pediatric burns	Orphan Drug designation Swiss Medic, EMA and FDA	Data not available	Bilayered artificial skin with epidermal layer composed of human epidermal cells and dermal fibroblasts on a hydrogel matrix	AT	NA	
Bilayered sheets	Epicel Cultured epidermal autografts	Vericel, Corp. (United States)	Deep dermal or full-thickness burns	US FDA 2007 October	50 cm ² sheets with a thickness of 2–8 cell layers	Cultured keratinocytes on murine 3T3 fibroblasts (each graft is attached to petrolatum gauze backing with titanium surgical grips)	AT	\$6000-\$10,000 per 1% TBSA	Administered to approx. 100 patients in the US annually (2018)

Bilayered sheets	Omnigraft Dermal Regeneration Matrix	Integra LifeSciences, Corp. (United States)	DFU	US FDA 2016 January	Sheets with two sizes of $4 \times 4 \text{ cm}^2$ and $7 \times 7 \text{ cm}^2$	Bilayered bioengineered scaffold, including an inner layer of bovine collagen and chondroitin, and an outer layer of thin silicone	Xn	\$499.00 per kit
Dermal matrix with fibroblasts	Dermagraft	Organogenesis Inc. (United States)	Full-thickness DFU > 6-week extended through the dermis without tendon, muscle, joint, capsule, or bone exposure	US FDA 2001 September	2 × 3-inch sheets	Fibroblasts on a piece of bio-absorbable scaffold	AL	\$1700 per treatment
Acellular dermal matrix	Aurix	Nuo Therapeutics, Inc. (United States)	All types of ulcers (DFU, pressure, VLU, etc.)	US FDA 2007 September	Depends on the size and condition of the wound	PRP hematogel	AL	\$430 per treatment
Acellular dermal matrix	Alloderm	Allergan			N/A	Acellular human allogeneic dermal matrix preserved by freeze-drying; can be used to prepare full-thickness wound bed before application of thin split-skin graft	AL	No data

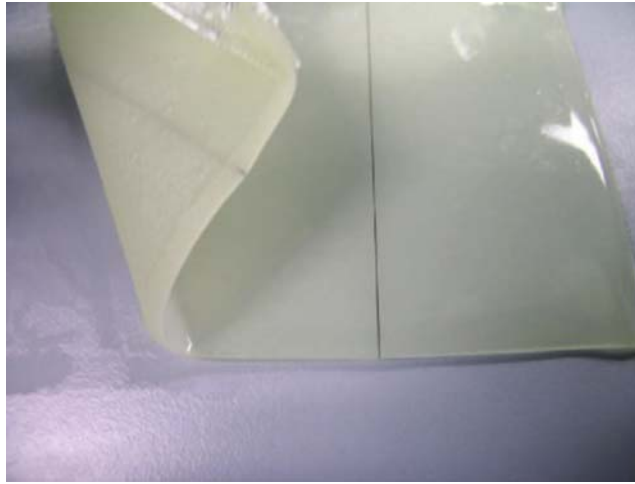
Continued

Table 15.5 Current tissue engineering methodologies for managing wounds and burns. *Continued*

Technology approach	Trade name/ Proper name	Manufacturer	Indications	Approved by/date	Dosage	Description	At/ AL	Price (US \$)	Usage
Acellular dermal matrix	Matriderm	Medskin Solutions/Dr Suwelack AG	Used in combination with autologous split-thickness skin grafts for reconstruction of deep-dermal defects and full-thickness skin wounds in plastic reconstructive surgery and in surgical treatment of burns, trauma and dermatological diseases, and DFU/chronic wounds	510(k) FDA 2021	210 × 297 mm sheet (1 mm or 2 mm thick)	Scaffold consisting of native bovine type I, III and V collagen fiber template incorporating elastin hydrolysate that is converted into native host collagen	NA	No data	
Skin cell suspension	KeraHeal	Biosolution, Co., Ltd. (South Korea)	Deep partial-thickness burns (>30% of TBSA) and full-thickness burns (>10% of TBSA)	South Korea MFDS 2006 May	1 mL skin-derived keratinocytes suspension/100–400 cm ²	Human skin-derived keratinocytes	AT	Approx. \$3561/100–400 cm ² per vial	Predicted to reach 450 patients in 2021
Skin cell suspension	KeraHeal-Allo	Biosolution, Co., Ltd. (South Korea)	Deep partial-thickness burns	South Korea MFDS 2015 October	One syringe (2.0 × 10 ⁷ skin-derived keratinocytes/1.5 mL) to the image area of 100 cm ²	Human skin-derived keratinocytes suspended in a thermo-sensitive hydrogel	AL	Approx. \$628 per 1.5 mL	Predicted to reach approx. 217,000 patients in 2021; occupied 20% of the market in 2017

Skin cell suspension	ReCell autologous cell harvesting device	Ativa medical	RES regenerative epidermal suspension for direct application to acute partial-thickness thermal burn wounds or application in combination with meshed autografting for acute full-thickness thermal burn wounds		N/A	Noncultured mixed cell population consisting of keratinocytes, fibroblasts, and melanocytes	AT	No data	
Dermal matrix with keratinocytes only	Holoderm	Tego science (South Korea)	Deep partial- and full-thickness burns	South Korea MFDS 2002 December	56 cm ² /piece consisting of 1–4 billion keratinocytes	Cultured keratinocyte sheet	AT	\$697.76 per cm ²	
Dermal matrix with keratinocytes only	Kaloderm	Tego Science (South Korea)	Deep partial-thickness burns and DFU	South Korea MFDS 2005 March (for burns) 2010 January (for DFU)	Each sheet contains $>2 \times 10^7$ cells backed by vaseline gauze. The amount is determined considering the size and condition of the wound	Cultured keratinocyte sheet	AL	\$26.5 per 1 cm ² for product with 56 cm ² units	300,000 sheets used since 2005
Electrospin matrix deposition device	Spincare	Nanomedic Technologies Ltd	Partial-thickness wounds and burns, hard to dress areas e.g., groin, hand face	Undergoing clinical trials	Ampule containing nanofibres	Electrospun nanofibrous polymeric bandages	NA		

Table adapted from Ramezankhani, R., Torabi, S., Minaei, N., Madani, H., Rezaeiani, S., Hassani, S.N., Gee, A.P., Dominici, M., Silva, D.N., Baharvand, H., Hajizadeh-Saffar, E., 2020. Two decades of global progress in authorized advanced therapy medicinal products: an emerging revolution in therapeutic strategies. *Front Cell Dev Biol* 8, 547653. <https://doi.org/10.3389/fcell.2020.547653>. Copyright 2020 by Creative Commons Attribution 4.0 International License.

**FIGURE 15.7**

Integra with the silicone upper layer in situ. A 10 cm $\times$ 12.5 cm of integra sheet is shown. From Anthony, E.T., Syed, M., Myers, S., Moir, G., Navsaria, H., 2006. The development of novel dermal matrices for cutaneous wound repair. *Drug Discov Today Ther Strat* 3(1), 81–86.

Matriderm

Matriderm is a product with a collagen base that includes elastin and no silicone surface layer. Although AlloDerm had been used as a single-staged grafting system, Matriderm has been the first to popularize this approach. Matriderm is engrafted to a full-thickness wound bed with an overlying thin split-thickness autograft, in the same procedure. This has significant cost advantages compared to two procedures separated by weeks, and it appears to provide acceptable graft take.

Biobrane

Biobrane is a thin bilayered dressing that aids the delivery of epidermal grafts by providing improved mechanical support and adhesion. It consists of a porous silicone outer layer bonded to an inert nylon fabric that is coated with peptides derived from porcine type I collagen to promote adherence. It is a very useful primary dressing for partial-thickness wounds in children that limits the need for painful dressing change, reduces in-patient stay, and lowers infection. In studies where preconfluent autologous keratinocytes seeded on Biobrane were applied directly to patients' donor and burn wound sites, healing times were comparable to keratinocyte sheets.²³

Spincare

Spincare is a new handheld device designed to deposit electrospun nanofibers including collagen rapidly onto a wound bed. The nanofibers have a high tensile strength, are flexible and breathable, forming a web-like mesh across skin, with particular application in hard-to-reach areas such as the hands, feet, and groin. It is currently undergoing clinical trials for partial-thickness burns and chronic wounds.

15.11.2 Dermal matrix with fibroblasts

A dermal template can be enhanced by a period of in vitro coculture with dermal fibroblasts. The addition of dermal fibroblasts to a collagen gel helps promote the deposition of ECM components such as additional collagen, hyaluronic acid, and chondroitin 4,6-disulfate.²⁴ These findings suggested that such a period of culture prior to implantation might have profound effects on the dermal equivalent eventually grafted; after 4 weeks in culture, the material possessed a more complex composition and, in many ways, more closely resembled normal dermis. Since that time, other benefits associated with in vitro coculture have surfaced and are summarized in Table 15.6. Dermal equivalents repopulated with cells may enhance dermal regeneration and improve both keratinocyte adherence and wound healing. Although the benefits of cellular skin substitutes have been reported, additional evidence suggests that these cellular substitutes may elicit proinflammatory macrophage responses that result in scar formation compared to acellular substitutes. Removing the cellular component may change the macrophage response to a more immunomodulatory and regenerative response, which facilitates constructive tissue remodeling.²⁵

Dermagraft

In contrast to naturally derived biomaterial scaffolds, **Dermagraft** comprises an absorbable polymer scaffold (polyglycolic acid or polyglactin-910) seeded with neonatal allogeneic fibroblasts (Fig. 15.8). An ECM consisting of collagens, tenascin, vitronectin, glycosaminoglycans, and growth factors is laid down by the fibroblasts, and this assembly remains biochemically active after cryopreservation while the provisional dermal matrix is resorbable. Dermagraft has been used more to stimulate wound healing in chronic wounds such as diabetic foot ulcers than resurface full-thickness acute burn wounds, but has been as successful as split-thickness grafts in the treatment of burns wounds excised to varying depths.²⁶

15.11.3 Skin cell suspensions (applied as sprays or injections)

Recell and Keraheal

An interesting alternative delivery method for keratinocytes for acute burn injuries involves the use of sprayed cell suspensions. Examples of these products include **Recell**, which uses freshly isolated autologous keratinocytes and was approved by the FDA in 2018, and **Keraheal**, which employs autologous cultured keratinocytes. Advantages of this approach include the rapid and easy delivery of cells, thereby avoiding handling of fragile epidermal sheets. In the case of cultured keratinocytes, the spray delivery harvests the cells when they are subconfluent in order to reduce culture time and maintain the cells in a proliferative, undifferentiated state.

Table 15.6 Reported benefits associated with in vitro seeding of dermal analogues.

Proposed benefits

- Improved collagen deposition and remodeling
- Improved DEJ formation
- Enhanced survival of keratinocytes
- Decreased myofibroblast formation
- Decreased wound contraction

**FIGURE 15.8**

Commercially available product Dermagraft. *From Advanced Biohealing.*

15.11.4 Dermal matrix with keratinocytes

Kaloderm and Holoderm

Kaloderm and **Holoderm** utilize a dermal matrix with keratinocytes seeded on top. The systems can be stored at -60°C before use. The key difference between the two products is the source of the keratinocytes. **Kaloderm** incorporates keratinocytes originally isolated from circumcised infant foreskin keratinocytes, whereas **holoderm** sources keratinocytes from adult hair follicles. The two systems have been approved for use in South Korea for treatment of partial-thickness burns. **Holoderm** is also approved for treatment of full-thickness burns.

Full-thickness skin equivalents

Bilayered skin substitutes, comprised of both dermal and epidermal components, are complex tissue equivalents and aim to provide more complete repair and regeneration of the skin. They typically consist of a collagen-based dermal matrix embedded with fibroblasts and an epidermal layer seeded on the surface. Cultivation of these constructs at the interface between culture media and the air promotes the terminal differentiation and stratification of the epidermis, which is essential for development of its barrier function. In early studies, the construction of a dermal collagen–glycosaminoglycan membrane seeded with autologous keratinocytes and cultured until maturation was successfully applied to full-thickness defects. This construct was then modified by incorporating autologous fibroblasts in the dermal layer and applied to four patients; of 13 grafts, nine took

successfully.²⁶ Later papers showed similar results and again less successful than the conventional autograft where take rates approach 100%. Of note, histological differences include lack of a normal blood vessel network in the dermis of the cultured skin substitute when compared to conventional split-thickness grafts and (or possibly subsequent to) a less keratinized epithelium.

Apligraf

Commercially available **Apligraf** is a full-thickness skin equivalent used to treat chronic diabetic and venous ulcers. It consists of bovine type I collagen matrix cultured with allogeneic male neonatal fibroblasts and keratinocytes, and the bilayered structure resembles normal human skin (Fig. 15.9). Apligraf

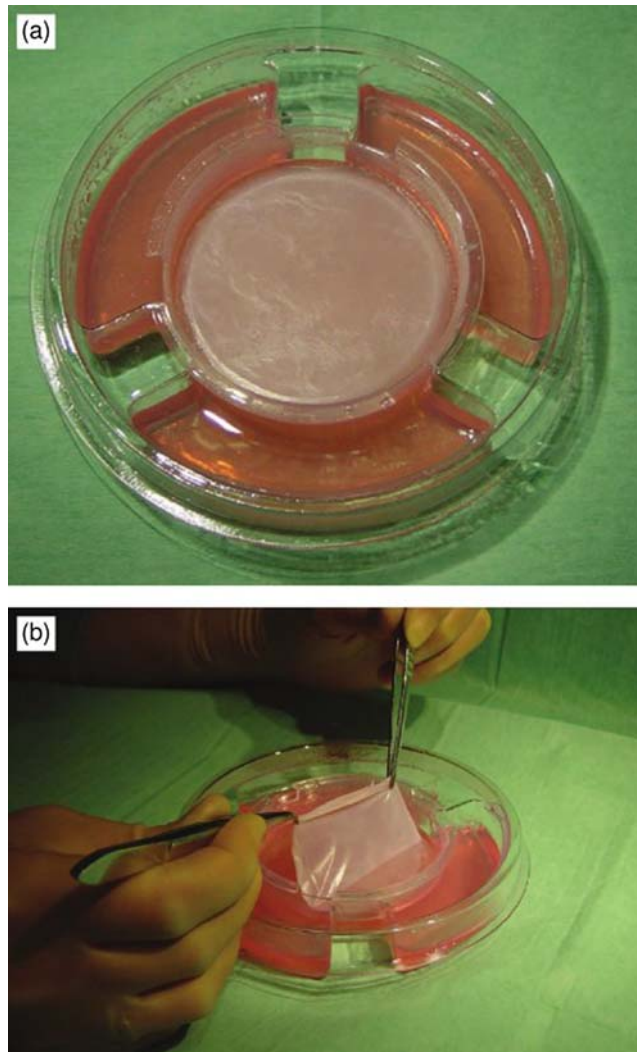


FIGURE 15.9

(a) Apligraf in transwell. (b) Apligraf being freed from agarose media prior to clinical application.

elicits a low immunogenic response, and this is thought to be ascribed to the lack of Langerhans cells. At the same time, this product was shown not to persist long term in a deep dermal wound model but instead acted as a temporary biological dressing, providing growth factors to the wound.¹⁴ Apligraf is known to have similar effects to meshed autograft when applied to partial-thickness donor sites with no signs of classical clinical rejection and when combined with autologous meshed split-skin graft, produces a more favorable result than conventional split-skin grafting alone. At present, Apligraf is licensed only for the treatment of diabetic foot and venous leg ulcers. In a recent clinical trial that elucidated the mechanisms by which Apligraf promoted healing of venous leg ulcers in patients treated with Apligraf in combination with standard care (compression therapy) or standard care alone, Apligraf-treated wounds displayed distinct transcriptome patterns suggesting a switch from a nonhealing to healing tissue response, akin to acute wound healing, involving keratinocyte activation, growth factor signaling, modulation of inflammatory signaling, and attenuation of Wnt/ β -catenin signaling.²⁷

DenovoSkin

DenovoSkin is a bioengineered system using autologous dermal fibroblasts and keratinocytes. A small skin biopsy is taken from the patient, skin cells are cultured and then transferred to a hydrogel membrane. It is currently undergoing clinical trials (Phase I for pediatric patients and Phase II for adults), has passed initial safety testing, and was granted Orphan Drug Designation by the EMA and FDA for the treatment of burns.

15.12 The use of adult stem cells in tissue-engineered skin

The ideal cell source to be used in tissue-engineered skin should be pluripotent, highly proliferative and expandable, readily available in abundance, easily accessible with minimal invasive procedures, and safe for autologous and allogeneic transplantation without provoking an immune response. Stem cells are receiving a lot of attention because of their transgermal and pluripotential nature, and significant progress has been made identifying them (see Chapter 2 Stem cells). The rationale for the potential use of adult stem cells in skin repair and tissue-engineered skin is discussed in the following section.

15.12.1 Induced pluripotent stem cells

Recent advances in technology have made it possible for differentiated, adult somatic cells to be reprogrammed to generate induced pluripotent stem cells (iPSCs), which exhibit similar characteristics to embryonic stem cells (ESCs). This can be achieved by exogenous addition of four transcription factors (Oct-3/4, Sox2, c-Myc, and Klf4) using retroviral transduction (see Chapter 2 Stem cells). iPSCs can generate a wide range of differentiated cell types. Investigations in recent years have shown that iPSCs are an attractive cell source, which can be used for skin tissue engineering and for regenerative medicine including therapy for skin wounds and disorders without any ethical or moral issues associated with ESCs.

Human iPSCs are capable of producing skin dermal stem cells, which can differentiate into neural and mesodermal cell lineages that can contribute to wound healing such as adipocytes, fibroblasts, and smooth muscle cells, and even induce keratinization when cocultured with human epidermal keratinocytes. iPSCs derived from keratinocytes are able to regenerate epidermis, hair follicles, and sebaceous

glands in the skin, and iPSC-derived folliculogenic human epithelial stem cells can reconstitute all hair follicle lineages and the IFE. These cells have also been used to generate human 3D skin equivalents comprised of iPSC-derived fibroblasts, keratinocytes, and melanocytes, which contain functional epidermal melanin units.

iPSCs have also been explored as a novel therapeutic modality for the devastating blistering skin disease recessive dystrophic epidermolysis bullosa (RDEB). Using specific experimental protocols to differentiate iPSCs into keratinocytes and dermal fibroblasts, the synthesis of wild-type collagen type VII was restored, thereby treating RDEB indicating their potential.²⁸ In recent work, the same authors demonstrated that combined use of iPSCs and CRISPR/Cas9-induced gene editing tools was effective in the treatment of RDEB and offered a safer approach in the establishment of gene and cell therapies for skin hereditary diseases for future clinical use.²⁹ These studies highlight the potential of iPSCs in the development of gene and stem cell therapy for skin disorders such as RDEB, tissue-engineered skin scaffolds to generate all cell types, components, and appendages of the skin for the treatment of wounds and providing useful in vitro models to study skin diseases.

Despite their therapeutic benefits, there are some unresolved safety issues with the use of iPSCs. These include associated cancer risk development through the use of retroviral vectors, inefficient cell reprogramming that yields low cell numbers, epigenetic memory retained from parent cells, genetic instability, and potential immunogenicity.⁴ New advances in technologies for obtaining iPSCs such as reprogramming of primary human skin fibroblasts using only CRISPR activators, which enable site-specific genome editing and reduces the risk of insertional mutagenesis, rather than traditional methods of retroviral transduction, will make the generation of iPSCs easier and more efficient to use for future applications. iPSCs hold great promise in the field of skin tissue engineering and regenerative medicine as autologous cells can be isolated from individual patients, thus circumventing any immunological or rejection issues while promoting patient-tailored treatment. At present, iPSC technology is primarily within preclinical stages, with early individual case studies where there is no alternative therapy. The application of iPSC technology for the treatment of RDEB is a remarkable example of the future potential of iPSCs.

15.12.2 Mesenchymal stem cells and adipose-derived stromal cells

Human mesenchymal stem cells (MSCs) are derived from the stroma of the bone marrow. They have also been isolated from other tissues such as nerve tissue, adipose tissue, umbilical cord blood, and dermis. Their isolation is relatively simple but indefinite propagation is difficult to achieve. **Adipose-derived stromal/stem cells (ASCs)**, a different progenitor cell type, can be harvested in abundance from lipoaspirate and discarded subcutaneous tissue and when grown in culture are easily expanded and retain their multipotency. The procedure has minimal risk and donor mortality. Both MSCs and ASCs are natural key players in tissue repair and regeneration and give rise to many cell types and tissues.

MSCs and ASCs play a role in wound healing in both acute and chronic wounds. This is achieved through paracrine secretion of growth factors and cytokines that promote angiogenesis, cell proliferation, migration, reepithelialization, ECM deposition, and turnover. In addition, MSCs exhibit antiinflammatory, antiscarring, antimicrobial, immune-modulative, and immunosuppressive properties

while ASCs exhibit antioxidative, angiogenic, and immunosuppressive properties. These cells can be delivered to the wounds either directly (e.g., through injecting, spraying, or systemic administration) or via skin scaffolds and dressings and have been shown to benefit wound healing clinically.

MSCs and ASCs release small extracellular vesicles called exosomes, which regulate many biological processes associated with wound healing process, such as cell proliferation, cell migration, reepithelialization, and blood vessel formation. These effects are mediated through important signaling pathways such as Akt/ERK/STAT3 and TGF- β /Smad, among others. Furthermore, MSC-derived exosomes induce the expression of growth factors and secrete growth factors such as insulin-like growth factor-1 (IGF1), nerve growth factor (NGF), hepatocyte growth factor (HGF) and stromal-derived growth factor-1 (SDF1) vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2 (FGF-2), hepatocyte growth factor (HGF), and platelet-derived growth factor BB (PDGF-BB), which promote wound healing.

Although MSCs are commonly used in preclinical and clinical studies, there is a lack of consistency in approach and analysis, making MSC technology still in early clinical trial (case studies, Phase I/II). The majority of Phase I and II studies have yet to publish their outcomes for treatment of wounds, burns, and diabetic ulcers. However, individual case studies do indicate promise in this area for MSC technology. Overall, the properties of MSCs and ASCs render them good candidates in tissue engineering.

15.13 Future perspective

From a small skin biopsy, we can currently isolate and expand enough keratinocytes in culture to cover the entire body surface area of an adult in 2–3 weeks. Unfortunately, this lengthy period is at odds with the current management of acute burns and is affected by the age of the patient. Moreover, the resultant sheets are difficult to handle, have variable take rates, and are highly fragile. Depletion of stem cells in current culture systems can also contribute to late failure of grafts after apparently successful grafting. There have also been major advancements in the engineering of full-thickness skin equivalents that closely mimic the dermal and epidermal compartments of skin, and these constructs are effective in treating chronic nonhealing wounds, such as diabetic ulcers. However, there are still major limitations in the ability to recreate more complex features of the tissue, such as hair follicles, glands, and blood vessels. Moreover, some skin equivalents may trigger immune rejection requiring additional interventional treatment. In addition, engineered skin is time-consuming and costly to produce, and the efficacy is highly variable. There are therefore significant opportunities for improvement in the functionality, reliability, and economics of engineered skin equivalents.

The development of next-generation skin equivalents will require a multidisciplinary approach, drawing on researchers with expertise in cell biology, clinical medicine, and engineering. At a cellular level, there is still much that we do not understand about the dynamic communication between different cell types within a wound environment, as well as the influences of aging, genetic variation, and underlying diseases. Thus, deeper insight into the mechanisms involved in the pathogenesis of burns and chronic wounds could provide insight into the essential cells and processes to target with tissue-engineered therapies. For example, recent “omic”-level profiling of the key genes, proteins, and metabolites, particularly at the single-cell level, could provide molecular-level detail on the pathomechanisms of

defective healing.^{30,31} Moreover, a refined understanding of the different types of wounds or burns and associated biomarkers could lead to more targeted and patient-specific therapies.

From a biomaterial's perspective, there is also much that can be done to create more sophisticated and bioactive constructs that promote healthy tissue healing. While collagen-based materials have been the mainstay of dermal scaffolds and templates, the ECM of native skin is a much more complex mixture of microfibrils, elastic fibers, and proteoglycans. Thus, incorporation of some of the key molecules into scaffolding biomaterials may lead to improved healing and regeneration. Likewise, advanced fabrication strategies to better mimic the micro- and nanostructure of the dermal ECM may further enhance cell migration, remodeling, and integration of engineering skin constructs.³² Finally, the incorporation of small molecules, growth factors, or RNA/DNA into biomaterial scaffolds offer the opportunity to target and modulate key healing processes that may be dysfunctional within the wound environment (e.g., excess inflammation).^{33,34} While scientifically promising, significant challenges remain for successfully translating these types of technologies into the clinic, including demonstration of efficacy beyond existing products, a complex regulatory approval process for such "combination" products,³⁵ and a potentially high cost of the therapies.

Engineering advanced skin equivalents with greater cellular and structural complexity, such as hair follicles and glands, also remains a major challenge. One of the main obstacles for recreating these structures *in vitro* is the ability to coculture multiple different cell types in the correct spatial organization while maintaining their specialized phenotypes. However, recent advances in biofabrication methods, such as 3D bioprinting, have opened up new opportunities for precision engineering of complex tissues. For example, 3D printed micromolds have been used to fabricate dermal matrices with microwells mimicking hair follicles.³⁵ When aggregates of dermal papilla cells, the specialized hair-inducing cells within the dermis, were then seeded within the wells and overlaid with cultured keratinocytes, the tri-culture promoted the differentiation and growth of hair follicles and hair. This study demonstrated for the first time the ability to grow human hair *in vitro*, purely from cultured cells, and emphasized the importance of how correct spatial interactions between cells, and the niche is essential for engineering specialized tissue structures. Ongoing studies employing technologies such as 3D bioprinting and organoid culture models are similarly attempting to improve the functionality of human skin equivalents and have made progress in incorporating vascular, pigmentation, and subcutaneous fat into the engineered skin constructs.^{36,37} As noted above, cost, safety, and efficacy will be key challenges in translating such technologies into the clinic, and the level of complexity of engineered skin equivalents should always be considered in the context of the specific application and what is required by the patient.

Summary

- Skin wound healing is a highly organized and complex series of processes comprising distinct but overlapping phases, which results in the restoration of tissue structure integrity and tissue functionality.
- Wounds are clinically divided into acute wounds such as burn injuries and chronic wounds such as diabetic or venous ulcers. Acute wound healing progresses in a timely and orderly pattern, whereas chronic wound healing is delayed. Care and management of these wound place a significant financial burden on the healthcare system. Alternative treatment approaches are needed to potentially improve wound healing.
- The structure of skin is very complex consisting of an epithelial and mesenchymal component. True tissue-engineered skin will need both these components and all the cell types.
- Great advances in skin substitutes have occurred over the last three decades. Skin is the first organ to be tissue-engineered. This began with the ability to culture and expand keratinocytes in 1975.
- It is now generally accepted that allogeneic keratinocytes do not survive transplantation and autologous cells are required for permanent closure. There is a role for allogeneic cells as a biological dressing to provide growth factors to the wound.
- A dermal component is required for optimal take and improved cosmesis. An understanding of dermoepidermal interactions developed over the years will allow for improvements in skin immunology, graft quality, and “take.”
- Tissue-engineered skin products are currently available as the epithelial component alone, as a template for dermal regeneration (with or without mesenchymal cells), or dermal component, or both epidermal and dermal components combined.
- Synthetic dermal replacements usually take the form of part of biological dressings. The true future of epidermal and dermal replacements lies in the advances of stem cell technology, identification of embryonic signaling pathways, biofabrication technology advancement, “Omic” technologies (genomics, transcriptomics, proteomics, and metabolomics), and taking a holistic approach to tissue-engineered skin based on the structure and function of skin.

Classical experiment

Keratinocyte stem cell therapy

Junctional epidermolysis bullosa (JEB) is a life-threatening, genetic skin fragility caused by mutations in genes that encode Laminin 332, type XVII collagen, integrin $\alpha 6 \beta 4$, or integrin $\alpha 3$, which leads to dermoepidermal tissue separation. Patients develop chronic skin wounds, which impact their life quality and are predisposed to skin cancer. Although no definite treatment exists for JEB, progress in developing cell- and gene-based therapies has been made through understanding of the cellular and molecular basis of JEB. In a recent study by Ref. 8, the integration of classical keratinocyte culture, clonal analysis, gene therapy with cultured epithelial autograft (CEA), to provide life-saving tissue engineering was demonstrated in a case of JEB (Fig. 15.10). In this study, a 7-year-old JEB patient, resulting in 80% total epidermal loss, was treated with corrected autologous transgenic keratinocyte stem cells transduced with laminin B3

(LAMB3) cDNA. The patient's keratinocytes were isolated from skin biopsy obtained from a nonblistering region, cultured, and transduced with a retroviral vector expressing the full-length LAMB3 cDNA under the control of the Moloney leukemia virus long terminal repeat (MLV LTR). The transduced keratinocytes were grown into CEA and grafted onto the patient. The corrected primary keratinocytes expressed functional laminin-332 heterotrimer proteins, and the blistering phenotype was fully reversed in skin grafts long term. Clonal analysis to assess the proliferative capacity and types of clones formed (holoclone, meroclone, and paraclone) in vitro and in vivo was performed. Almost complete epidermal regeneration of the entire epidermis was achieved after 1 month, with the regenerated epidermis demonstrating normal healing, elasticity, and restored dermis–epidermis junction. Patient skin biopsies taken at 1, 4, 8, and 21 months found transduced meroclones and paraclones were lost at 4 months

Classical experiment—continued

but holoclones persisted with seemingly unlimited proliferative capacity to maintain the epidermis, enabling the patient to be discharged.⁸ This treatment of a life-threatening genetic skin disease showcases the

importance and utility of skin tissue engineering and demonstrates how historic breakthroughs in skin culture have culminated in advanced therapy.

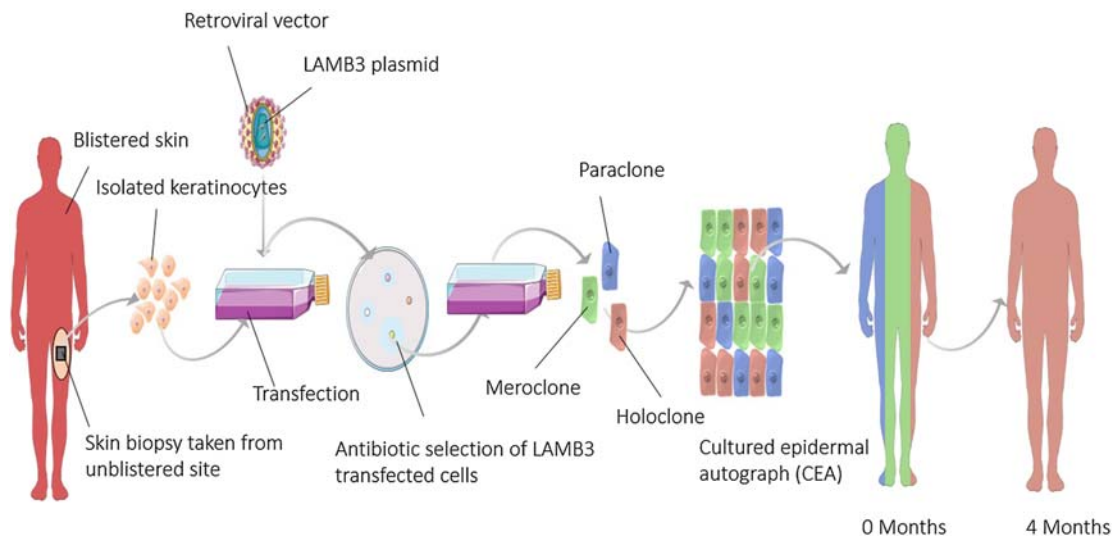


FIGURE 15.10

Skin tissue engineering creating a genetically modified cultured epidermal autograph to treat a 7-year-old child with junctional epidermolysis bullosa. Adapted from *Servier Medical Art, Creative Commons Attribution 3.0 France*. Hirsch, T., et al., 2017. *Regeneration of the entire human epidermis using transgenic stem cells. Nature 551(7680), 327–332.*

Continued

State-of-the-art experiment

Tissue-engineered skin

In a recent study by [Ref. 35](#), functional hair follicles were engineered within a skin equivalent model using entirely cultured cells. The engineered hair follicles were generated through the correct anatomical patterning of specialized hair inducing cells known as the dermal papilla. These cells exist as aggregates at the base of hair follicles and secrete soluble factors that regulate the growth and differentiation of hair follicle keratinocytes into hair cells. In this

experiment, the authors employed 3D printing to fabricate a micromolded insert, which was then placed onto a fibroblast-laden collagen gel while it set ([Fig. 15.11](#)). When removed from the gel, the micromolded insert left behind microwells mimicking the size of hair follicles. Cultured dermal papilla cells were then seeded into the base of the wells as aggregates, and finally, cultured keratinocytes were seeded onto the top portion of the wells and surface of the construct. Over a period of 3 weeks,

Continued

State-of-the-art experiment—continued

the keratinocytes began to differentiate into hair follicle cells and produced a mature hair that extended from the construct. In addition to engineering the first fully functional hair follicle in vitro, this study demonstrated the

importance of combining specialized cell types in the correct physiologic pattern for generating complex tissue structures, such as the hair follicle.

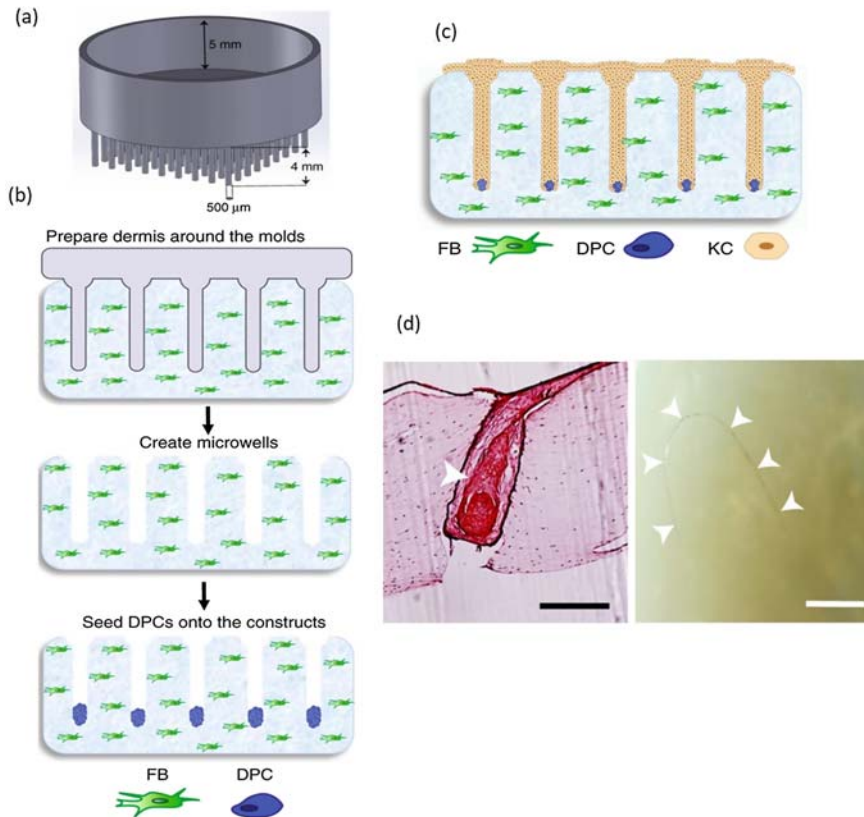


FIGURE 15.11

3D skin model with engineered functional hair follicles made entirely from cultured cells. (a) Hair follicle mold. (b) Fibroblasts (FB) and dermal papilla cells (DPC) seeded into constructs. (c) Keratinocytes (KC) seeded on top of construct. (d) Mature hair produced in construct. Adapted from Abaci, H.E., Coffman, A., Doucet, Y., Chen, J., Jacków, J., Wang, E., Guo, Z., Shin, J.U., Jahoda, C.A., Christiano, A.M., 2018. Tissue engineering of human hair follicles using a biomimetic developmental approach. *Nat. Commun.* 9(1), 5301. <https://doi.org/10.1038/s41467-018-07579-y>. Copyright 2018 by Creative Commons Attribution 4.0 International License.

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15.15 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter.
1. What are the phases of wound healing?
 2. Name the five cell types that form the epidermis and their key function.
 3. List the layers of the epidermis and how the shape of keratinocytes changes with differentiation throughout the layers.
 4. What keratins are associated with keratinocyte differentiation, and which keratins would you expect to see upregulated in an inflammatory skin disease?
 5. There are two distinct niches of dermal fibroblasts, where are they located in the skin and what is their key function?
 6. Which cell colonies are best for expanding keratinocytes; holoclones, meroclones, or paraclones, briefly explain why?
 7. List five key components of the dermoepidermal junction.
 8. Briefly describe the culture method that triggered a breakthrough in keratinocyte monolayer culture.

9. What is the Hayflick limit and why is it important in cell culture?
 10. List three types of dermal matrices used to create 3D skin cultures.
 11. Outline the different approaches to managing a burn when a split-thickness skin graft is not appropriate.
 12. What skin features are currently missing from cultured epidermal autografts?
 13. What are the key cells that contribute to allograft keratinocyte transplant rejection? Briefly outline their main function.
 14. List three naturally derived biomaterials currently used in engineered skin equivalents.
 15. Define the term “dermal template” and briefly describe the clinical application.
 16. Describe one limitation of cultured epidermal sheets overcome by keratinocyte spraying technologies.
 17. What is the definition of a stem cell and where are the stem cells found in the skin?
 18. Briefly describe the various types of models that have been proposed on how stem cells contribute to maintaining epidermal homeostasis.
 19. What are the main benefits of using adult stem cells in skin tissue engineering?
 20. What are some of the unresolved safety issues with the potential use of iPSCs for therapeutic application?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations.
1. Describe in detail the phases of wound healing and explain the differences between acute and chronic wound healing.
 2. Describe the epidemiology, causes, types, and classifications of burn injuries.
 3. Explain how keratinocytes are derived from epidermal stem cells and form the stratum corneum. What elements of this process are utilized in keratinocyte culture and why?
 4. Describe the process that triggers transplant rejection of allogeneic skin and what methodologies are being used to overcome this reaction?
 5. Illustrate how the basement membrane connects the epidermis to the dermis.
 6. Describe in detail one example of a tissue-engineered skin equivalent and its specific application. Discuss the advantages and disadvantages of the engineered tissue of traditional grafting procedures.
 7. Drawing upon basic concepts in tissue engineering and the current state-of-the-art in skin equivalent technology, propose a strategy to engineer skin constructs with functional sweat glands.
 8. How could skin culture be improved for managing burns patients and why might these additional features be important?
 9. Describe the main types of adult stem cells that can potentially be used in therapeutic application? Discuss the advantages and disadvantages of each.
 10. Describe in detail the evolution of epidermal replacement starting from keratinocyte monolayer culture using different culture methods to the development of keratinocyte sheets used in the treatment of burn wounds. How have “take” rates of keratinocyte sheets improved over the years

Challenge-based learning

Engineering hair follicles in tissue-engineered skin

Note for teachers: A CBL user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Tissue-engineered skin (TES) is a useful and effective product for wound repair and regeneration. The first-documented bioengineered skin product appeared 30 years ago as cultured epithelial autografts and has been used successfully since to treat major burns and other skin wounds. Even though TES substitutes are being widely used, there is room for improvement. Ideally, a TES product should completely replicate the normal physiology and function of the skin including appropriate mechanical properties, presence of hair follicles with normal hair growth cycle, sweat glands, and nerves. It should integrate with the host tissue with negligible scarring and induce minimal inflammation at the graft site. The long-term vision is to generate tissue-engineered skin with all the appendages that can be encountered in the human counterpart.

Motivation and stakeholders

Skin appendages (hair follicles, sebaceous and sweat glands) and nerves are important constituents for fully functional skin. Many cell types that constitute skin appendages can be produced using stem cells. The functionality of these appendages has been demonstrated in vitro and in vivo. Solutions to produce functional TES products including appendages should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as patients with skin burns, dermatologists, and biomaterial engineers.

Problem definition

Even though hair follicles have been successfully generated in the lab using mouse models, effective implementation as a treatment option is still not possible in humans due to several technical hurdles. First, the technical

complexity in isolating relevant cell types. Second, the difficulty to coculture different cell types in the correct spatial organization while maintaining their specialized phenotypes. And finally, difficulty in maintaining cycles of growth and rest in engineered hair follicles due to inability of the transplanted cells to regain their fully differentiated potential.

Challenge

The goal of this challenge-based learning (CBL) module is to design a novel strategy to overcome one of the challenges defined in the problem definition.

Learning framework

Reading of the Skin Tissue Engineering chapter and related literature will help you to understand

1. The function of the skin and its appendages.
2. The stages of hair growth.
3. Cell types needed to engineer hair follicles.
4. Cellular signals (both mechanical and chemical) needed to generate hair follicles during embryonic development.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

5. Biomaterials suitable for generation of tissue-engineered skin.
6. Current strategies to engineer human TES with hair follicles
7. Steps involved in the reconstruction of hair follicle in TES substitutes.
8. Cytokines and cell secretions needed to stimulate hair follicle growth in TES substitute.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

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15.16 Glossary

Acute wounds are a recent or sudden injuries before undergoing normal wound healing process.

Adaptive immunity is immunity acquired because of activated immune cells following exposure to antigen(s) in an immunocompetent individual.

Adipose-derived stromal/stem cells are adherent multipotent cells harvested from adipose tissue, expressing classical mesenchymal markers and able to differentiate into cells of mesenchymal lineage (adipocytes, osteocytes, and chondrocytes).

AlloDerm is an acellular matrix derived from human cadaver skin, used as a scaffold for tissue regeneration.

Allogeneic is derived from antigenically dissimilar individuals from the same species.

Allografts are grafts derived from antigenically different individuals of the same species.

Apligraf is a bioengineered skin substitute to treat chronic wounds. Contains an epidermal layer of human keratinocytes and a dermal layer composed of human fibroblasts in bovine collagen scaffold.

Appendages are outgrowths from an organism's body such as limbs, nails, and hair.

Autografts are transplanted tissues from the same individual.

Basement membrane zone is a dynamic interface between epidermis and dermis, consisting of structural proteins.

Biobrane is a temporary covering to facilitate wound healing following skin injury (burns, tissue grafts) not involving the dermal layer.

Biomaterial scaffolds are natural or synthetic substances used either to replace or repair injured tissue/organs.

Bulbous pemphigoid antigen is a member of plakin family of cytolinker proteins, connects keratin intermediate filaments and hemidesmosomes within keratinocytes to maintain structural integrity of the cell.

Burn wounds are distinct from other skin tissue injury involving hyperpermeability of capillaries leading to fluid leakage.

Chronic wounds are tissue injuries of the skin and soft tissues that fail to progress through the stages of a normal healing process.

Coagulation is a process in which a liquid is converted into a semisolid or solid state.

Collagen IV is a member of the collagen superfamily of proteins present exclusively in the basement membrane of the skin to provide a scaffold for other proteins.

Collagen VII is a major component of the anchoring fibrils, which provide stability to the dermal–epidermal adhesion.

Cultured epithelial autograft comprises keratinocytes harvested and cultured from a patient's own skin for use as skin grafts.

Cytoskeleton is a dynamic and complex network of filaments and tubules composed of actin, tubulin, lamina, keratin, and other proteins providing support for cell shape and movement, cellular signaling, and division of cells.

Deep dermalized dermis is acellular dermis derived from cadaver skin with the epidermis and all cellular components chemically removed.

Deep fibroreticularis is a layer of basement membrane in continuity with the connective tissue.

Deep partial skin loss is skin loss including the epidermis and papillary and reticular dermis caused by burns.

Dermagraft is a sterile, cryopreserved, human fibroblast–derived dermal substitute generated by the culture of neonatal dermal fibroblasts onto a bioresorbable polyglactin mesh scaffold.

Dermal matrix is decellularized tissue processed to remove all epidermal and dermal cells to obtain a bioactive scaffold used as graft to repair and regenerate skin.

Dermal papillae is a population of mesenchymal stem cells that reside just under the hair follicle, which stimulate hair follicle stem cells proliferation.

Dermal regeneration templates is a two-layer skin regeneration system composed of collagen-glycosaminoglycan scaffold to serve as an extracellular matrix analog to induce sequential regeneration of dermis, basement membrane, and epithelial layers.

Dermal templates are biologic or synthetic scaffolds that provide support for regeneration of complete skin layers.

Dermis a layer of skin between epidermis and subcutaneous tissue.

Dermo-epidermal junction is an area of tissue that joins the epidermis and epidermal layers of skin, composed of hemidesmosomes, anchoring filaments and fibrils, and type VII collagen.

Desmosomes are protein complexes present in the cytoplasm of a cell, responsible for cell–cell interaction and maintaining the mechanical integrity of the tissue.

- Diabetic foot ulcer** is a full-thickness skin wound that occurs in diabetic patients due to peripheral neuropathy associated with diabetes.
- Epidermal sheets** are cultured epithelial cells prepared in sheets for use as skin grafts during the treatment of large area burns.
- Epidermis** is the outermost epithelium covering of the skin composed of multiple keratinocytes-filled layers and an innermost melanocyte-filled basal layer next to the dermis.
- Extracellular matrix remodeling** is qualitative and quantitative changes in extracellular matrix mediated by specific enzymes.
- Fibroblasts** are spindle-shaped cells of mesenchymal origin that synthesize extracellular matrix proteins and collagen to provide tissue integrity.
- Fibronectin** is a high-molecular-weight glycoprotein found in the extracellular matrix playing a vital role in tissue repair and regeneration.
- Fibroplasia** is the formation of fibrous tissue during the wound healing process.
- Foreign body response** is inflammatory response to artificial organs, medical devices, or biomaterial in a host.
- Full-thickness grafts** are skin grafts that consist of complete epidermis and dermis.
- Full-thickness skin equivalents** is a tissue-engineered model of human skin generated as an alternative method to animal testing.
- Full-thickness skin loss** is the damage to both the epidermal and dermal layers of the skin exposing cutaneous tissue.
- Gene therapy** is a therapeutic strategy that focuses on genetic modification of cells to modify or manipulate gene expression to alter a biologic response.
- Glycosaminoglycans** are long and linear, negatively charged polysaccharides with repeating disaccharide units present in every mammalian tissue.
- Good clinical practice** is an international quality standard to provide a framework of principles that ensure safety of participants in a research study and validity and integrity of the collected data.
- Green sheets** are cultured epithelial cells prepared in sheets for use as skin grafts during the treatment of large area burns. The culture technique was developed by H. Green and colleagues in the 1970s.
- Hayflick limit** is the number of times a normal, somatic cell can multiply before cell division stops and the cell enters senescence.
- Hemidesmosomes** are specialized protein complexes present in epithelial cells that help in attachment of epithelial cells to the basement membrane.
- Human mesenchymal stem cells** are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts, chondrocytes, myocytes, and adipocytes.
- Immunological rejection** is an organism's reaction aimed to destroy the transplanted organ or tissue.
- Induced pluripotent stem cells** are a type of pluripotent stem cells that can be generated directly from somatic cells.
- Innate immunity** is one of the two main immunity strategies found in vertebrates, consists of physical, chemical, and cellular defenses against foreign antigen.
- Integra** is a double-layer dermis regeneration complex. Acting as the skin's epidermis and a scaffold for dermal skin cell regeneration.
- Intermediate densa** is an electron-dense component of the basement membrane zone between the epidermis and dermis of the skin, consists of type IV collagen, anchoring fibrils made of type VII collagen, and dermal microfibrils.
- Keraheal** is grafting rate enhancer composed of cultured human epidermal keratinocytes.
- Keratinocytes** are the primary type of cells found in the epidermis.
- Keratins** Intermediate filament proteins of epithelia containing molecular diversity to make up scales, hair, nails, feathers, horns, claws, etc.
- Lamellar (Pacinian) corpuscles** nerve endings in the skin that function as rapidly adapting, low-threshold mechanoreceptor sensitive to vibration and pressure.
- Laminin** Protein network in the basement membrane, which provides cell and tissue support and acts as a platform for complex signaling.
- Langerhans cells** Macrophages of the skin.
- Lucida** is an approximately 40-nanometer-wide electron-lucent zone between the plasma membrane of the basal cells and the lamina densa.

Melanocytes are melanin-producing neural crest-derived cells located among various cell types and primarily responsible for skin color.

Merkle cells are vital mechanoreceptors for light touch sensation.

Niche is a microenvironment within a specific anatomic location responsible for cell fate regulation.

Papillary dermis is the uppermost layer of the dermis, composed of fine and freely arranged collagen fibers.

Pressure ulcers are traumas to the skin and underlying tissue caused by prolonged pressure on the skin.

Proteoglycans are proteins that have one or more covalently attached long linear polysaccharides.

Recell is a device that enables skin sample procession into a cell suspension to treat acute thermal burns.

Reticular dermis is a dense irregular connective tissue, which gives the skin its overall strength and elasticity.

Rhinoplasty is a plastic surgery procedure for altering and reconstructing the nose.

Ruffini corpuscles are slowly adapting mechanoreceptors located in the cutaneous tissue between the dermal papillae and the hypodermis.

Skin equivalents are bioengineered tissue substitutes.

Skin replacement products or skin substitutes are a heterogeneous group of biological, synthetic, or biosynthetic materials for temporary or permanent open wound protection.

Spincare is a single-patient polymer-based sterile solution that enables fast and less painful healing through real-time printing of a localized nanofibrous matrix directly onto a wound.

Split-thickness is a graft containing the epidermis and a fraction of the dermis.

Stem cells are undifferentiated or partially differentiated cells that can differentiate into various types of cells and proliferate indefinitely to produce more of the same stem cell.

Stratum basale is the deepest layer of the epidermis containing dividing cells.

Stratum corneum is the outermost layer of the epidermis, which serves as an initial barrier against pathogens in the organism.

Stratum granulosum is a thin layer located between stratum corneum and stratum spinosum containing keratohyalin granules. In sole and palm skin, stratum granulosum is located between stratum lucidum and stratum spinosum.

Stratum spinosum is located between stratum granulosum and stratum basale and initiates keratinization process.

Subcutaneous tissue or hypodermis is the innermost layer of skin, consists of fat and connective tissues to host larger blood vessels and nerves while insulating to help regulate body temperature.

Superficial partial skin loss is partial-thickness loss of dermis presenting as a shallow open ulcer with a red, pink wound bed without slough.

T cells are white blood cells of the adaptive immune system.

Terminal differentiation(-ing) (maturation) is the final differentiation step of epidermal keratinocyte where a special mode of programmed cell death results in formation of corneocytes (rigid cell remnants without intracellular organelles).

The integumentary system is a set of organs including skin, hair, nails, and exocrine glands forming the outermost layer of an animal's body to protect and maintain the internal environment.

Type I and type III collagen are two major collagen types found in the body tissues. Both play a key role in the formation of the extracellular matrix, characterize tissue's tensile strength, and influence cell attachment and migration.

Venous leg ulcers are wounds that are thought to occur due to improper functioning of venous valves.

Wound contracture is physical deformity characterized by skin constriction and restrictions of functions during tissue recovery.

Wound healing is the mutually coordinated series of biochemical processes aimed at tissue restoration.

Xenografts are transplants of an organ, tissue, or cells to an individual of another species.

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Cartilage and bone regeneration

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16.1 Learning objectives

After reading this chapter you will be able to:

- Recognize the developmental aspects of bone and cartilage including joint development.
- Indicate the role and function, limitations, and applications of cartilage and skeletal stem cells.
- Understand repair capacity of articular cartilage and bone and to acknowledge that there is an inherent difference in the repair capacity among individuals.
- Understand the different types of bones and the functions of bone.
- Indicate the different biomaterials available and their use and limitations in skeletal regeneration.
- Detail the role of growth factors in bone repair.

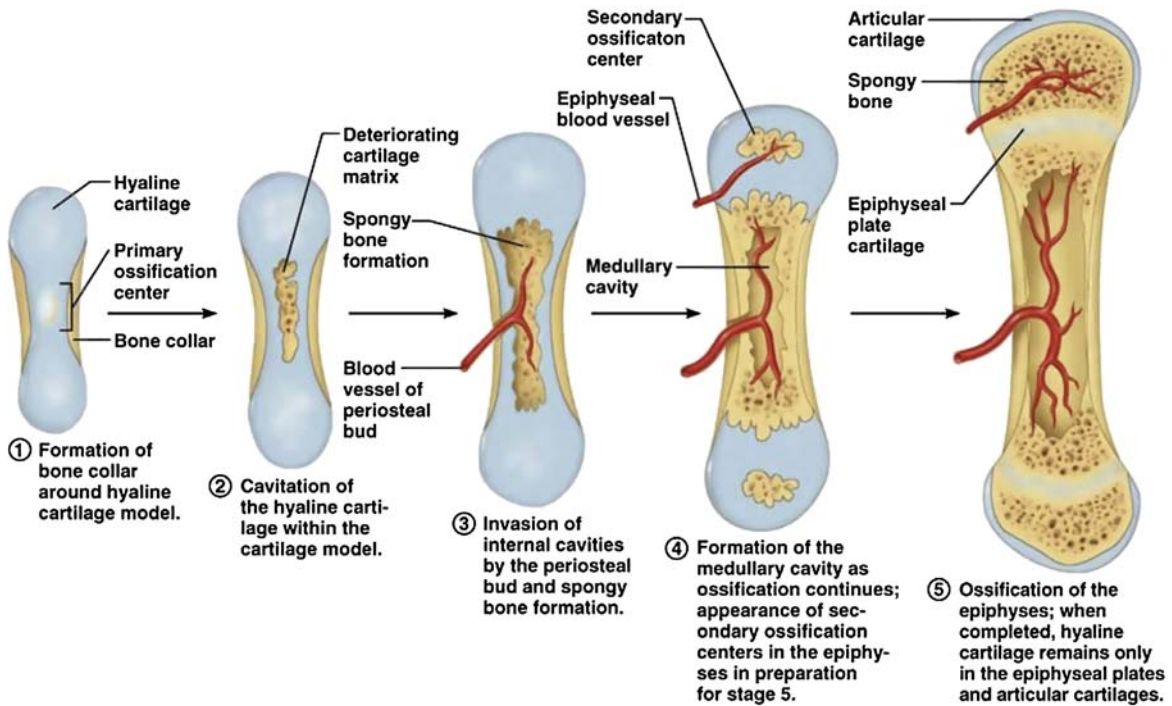
“If we consult the standard Chirurgical Writers from Hippocrates down to the present Age, we shall find, that an ulcerated Cartilage is universally allowed to be a very troublesome disease ... when destroyed, it is never recovered.”

Hunter, 1742

16.2 Introduction: cartilage

Cartilage tissue is derived from the mesoderm and gives rise to three different types of cartilages where the properties are dependent on the cells (the chondrocytes) and the extracellular matrix (ECM) produced by the chondrocytes: (1) hyaline cartilage is found in joints, rib cartilage, nose, trachea, and larynx; (2) elastic cartilage is found in the ear, epiglottis, and larynx; and (3) fibrous cartilage is found in intervertebral discs. The long bones initially consist of hyaline cartilage, and ossification gradually takes place in ossification centers where cartilage is gradually replaced by bone (Fig. 16.1) as presented later in this chapter.

After bone formation, hyaline cartilage remains at the end of long bones and in the epiphyseal plate cartilage in the ends of long bones. During longitudinal bone growth, the hyaline cartilage of the

**FIGURE 16.1**

The steps of cartilage and bone formation.

epiphyseal growth plate constantly adds new cartilage that is gradually replaced with bone and thus length is added until the end of adolescence in humans. In most primates, the epiphyseal growth cartilage is ossified in adulthood.

The cartilage of mesoderm origin is not the only cartilage formed in the body. The formation of the craniofacial skeletal elements and the jaw in particular was an important step in the evolution of higher vertebrates. Most facial bones and cartilage are generated during embryonic development by cranial neural crest cells (NCCs) that are a transient, multipotent population of cells that subsequently delaminate from the dorsal tip of the neural tube. The cells migrate along the long axis of the embryo and give rise to distinct populations. One of these cell types are the cranial NCCs that migrate to the level of the first somite and give rise to cartilage and connective tissues of the craniofacial region.¹

16.3 Cellular structures and matrix composition of hyaline cartilage

Embryonal cartilage gradually matures to meet the needs and demands of the adult organism. The initial high cell-to-matrix ratio (30% cells) in the embryo is reduced to approximately 2% cell content in the adult cartilage. The ECM consists of water (65%–80%), and the rest is solid material (20%–35%). Of the solid part, about 60% is collagen, 30% proteoglycans, and 10% comprises other proteins. The mechanical function of proteoglycans is to act as shock absorbers, whereas collagens

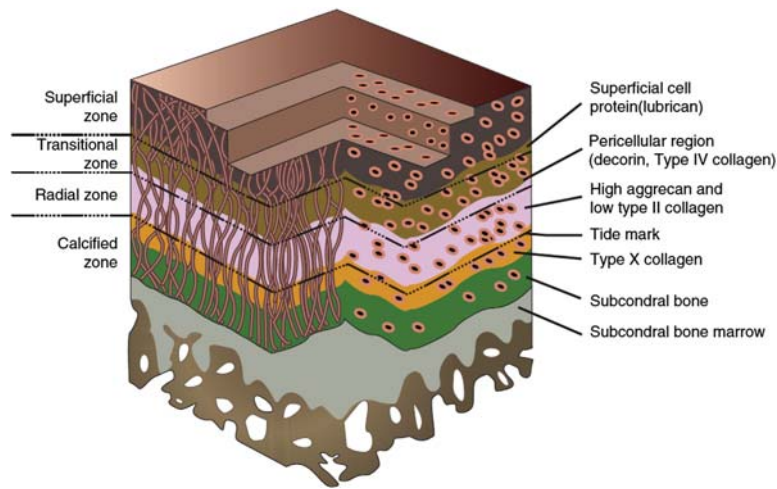


FIGURE 16.2

Structural and functional zones of articular cartilage. Adapted from Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Lavery S. *Composition and structure of articular cartilage: a template for tissue repair*. Clin Orthop. 2001:S26–S33.

provide resistance to shear stress. The average thickness in loaded areas of the human hyaline cartilage is 2.4 mm.

In young cartilage, only two zones can be identified in **articular cartilage**: the superficial and growth zones in contrast to mature cartilage where four structurally different zones with different functions and protein composition can be identified: the superficial, transitional, radial, and calcified zones (Fig. 16.2).² The superficial zone consists of elongated and flat cells embedded mainly in type I collagen, the zone lines the synovial joint, and its major component lubricin or superficial zonal protein functions as a lubricant and is normally only found in this area of the articular cartilage.

In the transitional zone, the cells are more rounded and pericellularly type IV collagen can be found. The major proteoglycan in this area is decorin. The collagen fibers in these first two regions are horizontally oriented. In the radial zone, chondrocytes are more sparsely distributed, and the major matrix proteins are the typical cartilage type II collagen that is radially oriented and the proteoglycan—aggrecan. Next to the subchondral bone, the cartilage becomes mineralized, and type X collagen is found in this area.

16.4 Collagen

The ECM in most tissues contains a number of related molecules that interact to form a structured network. The major protein in cartilage is the fibril forming type II collagen that gives structure and tensile strength to the cartilage. Collagen is defined as a structural protein of the ECM, and it contains one or more domains having the conformation of a triple helix.³ Collagen fibrils are composed of aggregates of five tropocollagens (three identical alpha II polypeptide chains assembled in a superhelix)

that have a diameter of 20 nm. This unique structure of the fibrils makes collagen insoluble and resistant to attack by degrading enzymes.

In the radial zone, the fibers are vertically oriented in a hexametric structure surrounding the chondrocytes; this thus explains the columnar appearance of chondrocytes typically seen in this zone. The predominant type of collagen is type II collagen, but small amounts of types I, V, VI, IX, X, and XI are also present. Collagens participating in quarter-staggered fibrils are of types II and XI; collagen-forming sheets have type X; collagen-beaded filaments have type VI; and fibrin-associated collagens are of type IX. The principal differences between collagens are outlined in [Fig. 16.3](#).

The turnover of collagens in adult normal cartilage is considered to be very slow but in certain disease states, the synthesis can be increased.

16.5 Proteoglycans

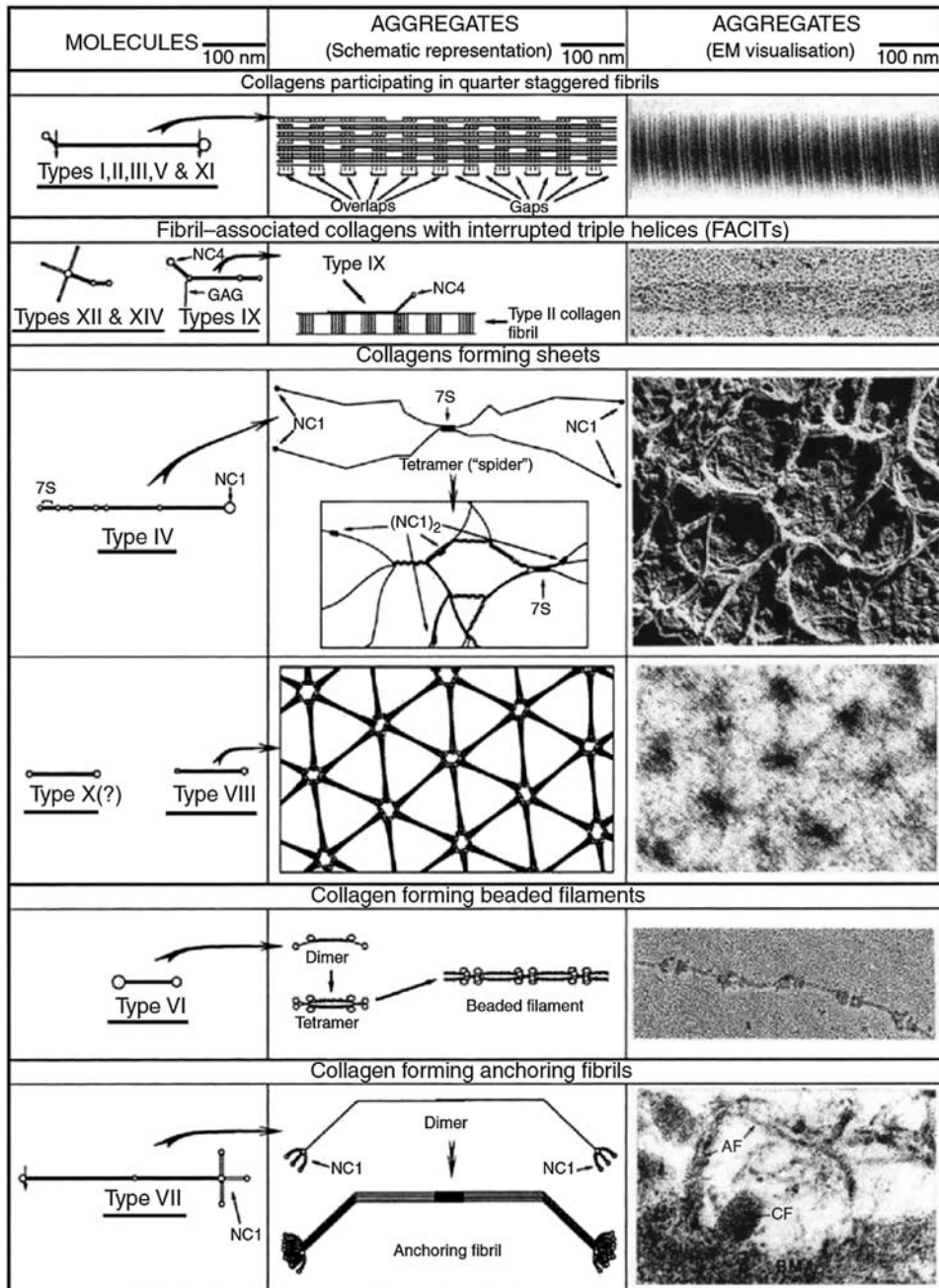
Two different types of proteoglycans are present in the articular cartilage: nonaggregating and aggregating. The smaller nonaggregating proteoglycans consist of a core protein to which a large number of sulfated glycosaminoglycans (GAGs) are attached laterally. The GAGs normally found in articular cartilage are keratan sulfate, chondroitin sulfate, and dermatan sulfate.

Larger aggregated proteoglycans are formed by the binding of smaller nonaggregating proteoglycans to hyaluronic acid, which has binding sites for core proteins. Hyaluronic acid is further bound to chondrocytes through the surface receptor CD44 ([Fig. 16.4](#)). The concentration of hyaluronic acid is higher during the embryonic formation of cartilage, and it has been shown that hyaluronic acids of certain molecular weights are **chondroinductive**.

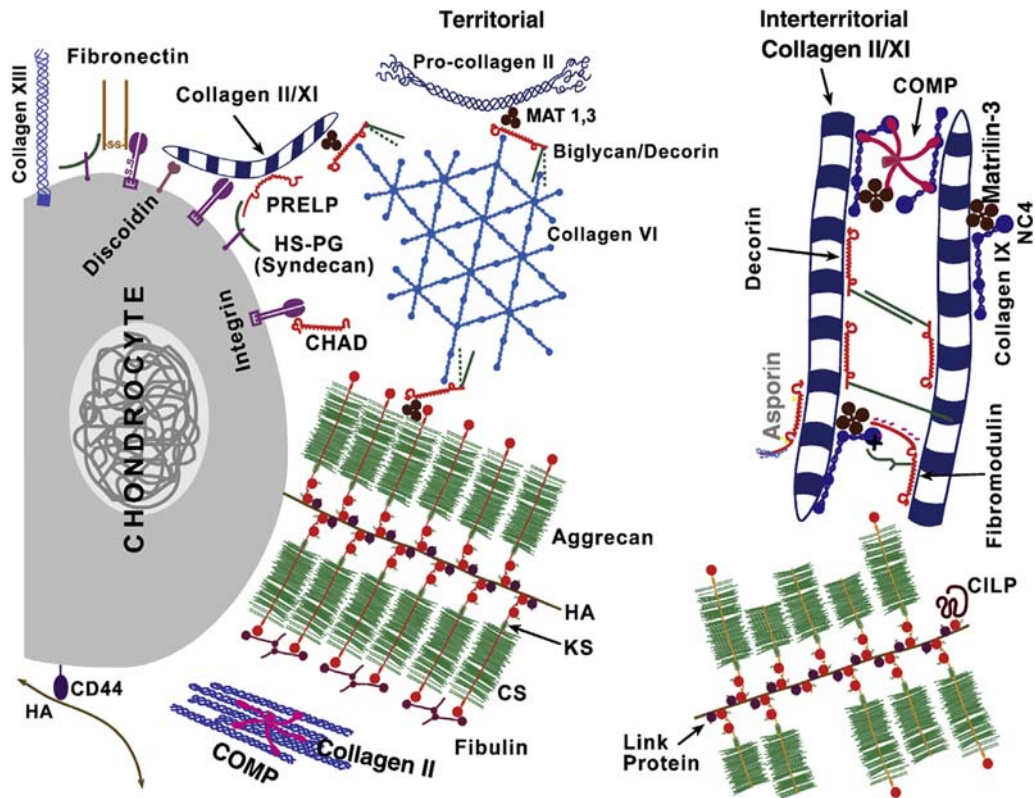
Within proteoglycans, negatively charged GAGs are packed around the core protein at a distance of 20–50 nm. Water molecules are bound to GAGs, and when the cartilage is compressed, water is retained within the matrix, which thereby limits the forces of compression. Decorin and biglycan both interact with collagens through the leucine-rich structures of their core proteins ([Fig. 16.4](#)).

16.6 The chondrocyte

The only cells existing in cartilage are the **chondrocytes**. These cells are characterized by their special ability to produce matrix and their highly developed cytoskeleton of actin filaments. The actin filaments contribute to the biomechanical properties of the chondrocytes. The interactions with the ECM are critical for both the development and maintenance of the cartilage. The cartilage matrix that surrounds chondrocytes in healthy articular cartilage is arranged into three zones depending on the distance from the cell ([Fig. 16.4](#)). The pericellular matrix lies immediately around cells, and in this microenvironment, molecules are located that interact with cell surface receptors, for example, hyaluronan binds the receptor CD44. Next to the pericellular matrix lies the territorial matrix, and further out is the interterritorial matrix. The types of collagens and the collagen-binding proteins that form the matrices are different in each zone. The **chondron** is defined as the functional unit of cartilage and consists of several chondrocytes and the immediate microenvironment of the surrounding pericellular matrix. The chondron also functions in insulating and physically separating chondrocytes from direct interaction with

**FIGURE 16.3**

Molecular structure and supramolecular assemblies of collagens. From Van Der Rest M, Garrone R. Collagen family of proteins. FASEB J. 1991;5:2814–2823.

**FIGURE 16.4**

The molecular organization of normal articular cartilage. From Heinegard D, Saxne T. *The role of the cartilage matrix in osteoarthritis*. Nat Rev Rheumatol. 2011;7:50–56.

the bulk of the load-bearing matrix. The number of chondrocytes per chondron varies from one to eight, with the lowest number being present in the superficial zone and the highest existing in the radial zone.⁴ The distribution of chondrocytes varies in different zones from approximately 7000 to 24,000 cells/mm³. From these figures, the number of chondrocytes beneath a certain area of an articular joint surface can be calculated to be approximately 24,000 cells/mm².

16.7 Stem cells in cartilage and proliferation of chondrocytes

Although one of the characteristics of articular cartilage is low cell turnover, there is evidence in the literature to show that proliferation may occur in healthy cartilage, OA cartilage, or in experimentally induced trauma. Generally, proliferating cells have been found either in superficial layers or in deep calcified layers. Due to the fact that proliferative cells are normally associated with some primitive features and stem cell properties, the findings of proliferative cells in these layers also raise the question as to the area from which the cartilage grows, that is, intrinsic or appositional. Based on cell tracing studies

in the developing joints of a marsupial (*Monodelphis domestica*), it was demonstrated that the progenitor cells of cartilage are located in the superficial layer, and one explanatory hypothesis is that articular cartilage grows appositionally.⁵

The anatomical structure surrounding the growth plate cartilage consists of a perichondreal ring of connective tissue, and the zone of Ranvier was demonstrated to harbor a stem cell niche where cells were demonstrated to migrate into the joint cartilage (a similar structure was also found in the vertebra of rabbits). The results have future implications in understanding the regenerative potential in the joint.⁶

16.8 Pathophysiology of cartilage lesion development

Cartilage has a limited capacity for regeneration. The poor repair capacity of adult cartilage is due to the total lack of innervation and vascular supply and that chondrocytes, even if they could respond to an injury, are trapped within the tight cartilage matrix.

In contrast to the adult cartilage, fetal cartilage has the ability to repair isolated lesions and ulcers (Fig. 16.5). An acute injury consisting of a relatively parallel incisional defect that did not violate any blood vessels (Fig. 16.5a). A photomicrograph obtained 3 days after the injury, showing dead chondrocytes adjacent to the incisional defect and beneath sites where the footpad of the ophthalmic knife came into contact with the cartilage (Fig. 16.5b). Seven days after the injury, the defect is completely filled. The site of the defect and the areas that came into contact with the footpads were hypocellular

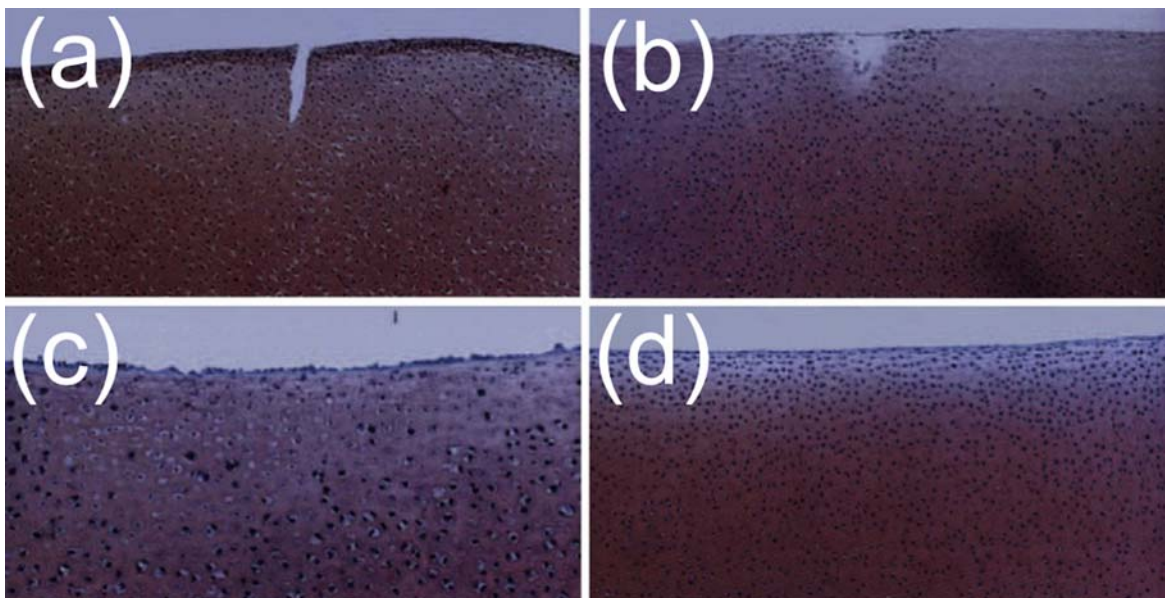


FIGURE 16.5

Spontaneous repair of superficial defects in articular cartilage in a fetal lamb model. From Namba RS, Meuli M, Sullivan KM, Le AX, Adzick NS. Spontaneous repair of superficial defects in articular cartilage in a fetal lamb model. *J Bone Jt Surg Am.* 1998;80:4–10.

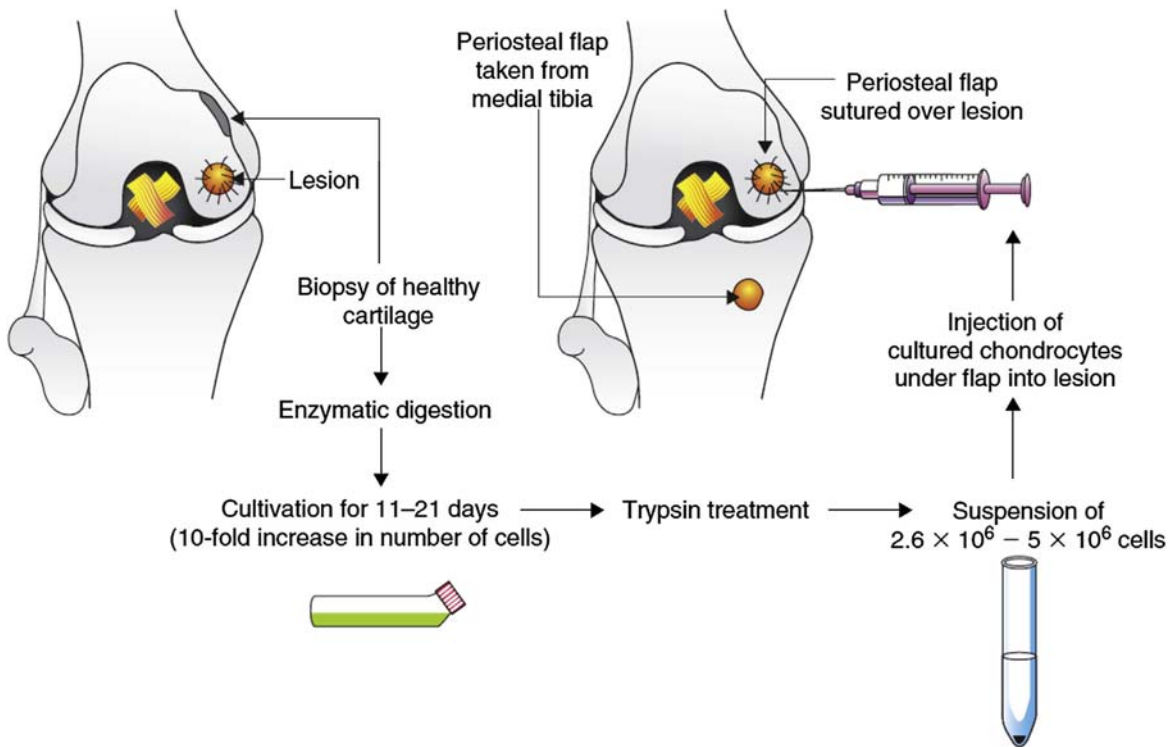
and contained sparsely populated chondrocytes. Increased mitotic figures were visible adjacent to the zone of injury (Fig. 16.5c). 28 days after the injury, the cellularity, matrix staining, and architectural arrangement of the cells matched those of the sham controls (Fig. 16.5d). This specific self-repairing ability could possibly be explained by the structural and compositional differences between adult and embryonic cartilage.⁷ Fetal cartilage contains higher amounts of hyaluronic acid and has a higher matrix-to-cell ratio. At the matrix level, the embryonic cartilage contains type I collagen and the GAG chondroitin-6 sulfate, while the adult cartilage mainly contains type II collagen and chondroitin-4 sulfate.

Acute or repetitive blunt joint trauma can damage articular cartilage and can result in the formation of isolated defects, and the remaining cells will be subjected to new mechanical forces that may result in cell death or apoptosis. The cellular processes ongoing on the rim of isolated defects have been compared to the process of the development of idiopathic **osteo-arthritis** (OA). It is therefore thought that even if smaller defects may self-repair, larger isolated lesions caused by trauma that are left untreated may progress into OA. OA is one of the most common forms of musculoskeletal diseases that affects millions of people throughout the world. The clinical symptoms of OA are pain and functional impairment that induce joint stiffness and dysfunction with subsequent impaired performance in daily life. As many as 25% of patients cannot cope with daily activities, and this often results in depression and social isolation. In the European Union and the United States combined, over one million joint replacements are performed each year as a consequence of OA. The main risk factors for OA are age, obesity, and any form of joint trauma where the limited repair capacity of articular cartilage is a confounding factor. OA is often thought of as a disease of the elderly; however, OA is evident in younger patients, with approximately 5% of the population between 35 and 54 years having radiographic signs of OA. Inflammation is a very important factor in the early event of OA and is probably initiated by the **traumatic injury** to tissue.⁸

16.9 Artificial induction of cartilage repair

The limited repair capacity of cartilage has forced researchers and surgeons to find methods to induce cartilage repair for decades. Several methods have been used where the general concept has been to use cells or tissues with chondrogenic potential. The most frequently used methods are bone marrow stimulations such as drilling or **microfracturing** of the subchondral bone to attract bone marrow cells. Chondrogenic tissue implants such as resurfacing with periosteum and perichondrium were popular in the 90s but very little used today. However, the repair tissue formed is usually of the fibrous cartilage type unable to withstand the joint load over time. Transfers of full cartilage tissues such as with multiple autologous osteochondral grafts and especially allogeneic osteochondral grafts are still in large use with a secure cartilage tissue substituting the destroyed cartilage area. However, the access is limiting and with allogeneic implants disease transmission is a risk.

The use of in vitro expanded chondrocytes for implantation has been used as an alternative method for cartilage repair since 1987. The **autologous chondrocyte implantation** (ACI) technique⁹ has developed into a clinical cell-based therapy for the repair of cartilage lesions worldwide. In this procedure, cartilage pieces are harvested arthroscopically from a healthy part of the joint, cells are isolated by enzymatic

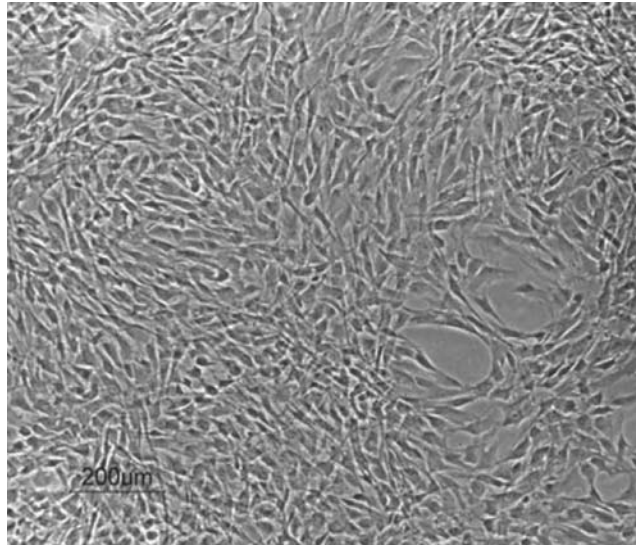
**FIGURE 16.6**

The procedure to transplant autologous chondrocytes.

digestion, culture expanded in vitro and finally implanted into the defect site at a high density and protected by either a periosteal flap or a collagen membrane (ACI Generation 1 and 2) (Fig. 16.6).

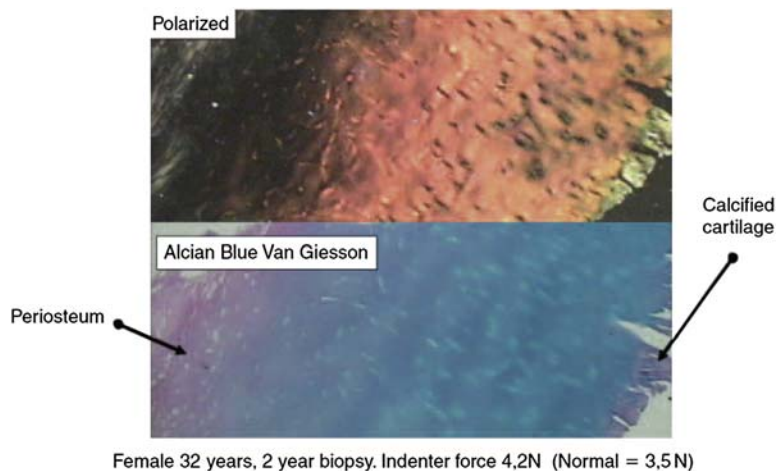
16.10 Rationale for cell implantation

Articular chondrocytes are cells responsible for the unique features of articular cartilage; therefore, it would appear appropriate to use true committed chondrocytes to engineer in vitro or in vivo cartilage to repair a cartilaginous defect. Human articular chondrocytes derived from articular cartilage biopsies have a limited proliferative potential that declines with age. Furthermore, chondrocytes grown in vitro in monolayer **dedifferentiate** and lose type II collagen production and their redifferentiation potential (Fig. 16.7). Expansion of human articular chondrocytes to be used for cell therapy is presently performed in the presence of 10% fetal calf serum or in autologous serum,⁹ and their redifferentiation in vitro and in vivo remains a challenge. However, if dedifferentiated chondrocytes are cultured in 3D cultures in gels as well as in porous scaffolds in the presence of chondrogenic inducing growth factors (e.g., TGF- β 1), the cells reexpress **phenotypic markers** of the articular chondrocyte.

**FIGURE 16.7**

The shape of human articular chondrocytes in a monolayer culture. These cells are then used in the transplanation procedures.

In a mesenchymal tissue defect site, the initial number of multipotential cells that can take part in the repair process is critical.¹⁰ The dedifferentiated chondrocytes have similarities to primitive mesenchymal cells, and a high-density cell implantation imitates the prechondrogenic cell condensation seen during limb formation and results in subsequent cartilage formation¹¹ (Fig. 16.8). Further, the clinical and functional long-term outcome of ACI remains high even after 10–20 years.¹²

**FIGURE 16.8**

Regenerated cartilage from the autologous chondrocyte transplantation procedure 2 years after cell implantation.

16.11 Cartilage specimens for implantation

Articular cartilage is composed of zones with different mechanical and functional properties; further, different locations in the joint have different cartilage properties, which raises the question of harvesting site for ACI. Extensive studies of the different layers of cartilage and the cartilage regeneration potential have concluded that full-thickness cartilage biopsies are needed for the tissue engineering of articular cartilage since the tissue formed by full-thickness chondrocytes accumulated the greatest amount of collagen, whereas the tissue formed by the mid-zone and deep-zone chondrocytes accumulated significantly more proteoglycans.¹³

16.12 Cell seeding density

The mean number of chondrocytes in human cartilage tissue is 9626 cells/mm³.⁴ With a mean height of the hyaline articular cartilage layer of 2.4 mm, a 1 cm² defect would have a volume of 24,000 mm³ and need approximately 2.4×10^7 cells. In vitro experiments with chondrocytes directly seeded to cartilage explants show a linear relationship between biosynthetic activity and the number of seeded chondrocytes.¹⁴ However, the number of cells needed for the implantation either as a suspension or in a scaffold has been studied extensively, although no consensus of seeding density has been reached. There are no published studies that evaluate the influence of cell-seeding concentration using human articular chondrocytes and clinically validated methods of culture in 3D. However, it is accepted that chondrogenesis is enhanced by increasing the cell seeding density and cell–cell interactions. However, the ideal number of cells needed to start and retain secure chondrogenesis is not known, although the goal of many commercial companies is to deliver 1×10^6 cells/cm².

The matrix used for cell transfer and differentiation influences chondrocytes. Matsiko et al.¹⁵ showed that relative larger porosities in a collagen-hyaluronic acid scaffold induced significantly higher cell proliferation, chondrogenic gene expression, and cartilage-like matrix deposition. Contrary in a polyurethane scaffold smaller pores stimulated chondrogenesis, while large pores induced instead a bone formation.

16.13 What type of chondrogenic cells is ideal for cartilage engineering?

Pure chondrocytes, epiphyseal or mature chondrocytes, and allogeneic or autologous chondrocytes have been used in similar processes; these include ear and nasal chondrocytes and autologous mesenchymal bone marrow stromal cells (BMSCs), as summarized in Table 16.1.

Table 16.1 Cells with chondrogenic potential.

Chondrocytes	MSCs
Articular	Bone marrow
Auricular	Adipose cells
Septal nasal	Periosteum
Costal	Synovial membrane

16.14 Allogeneic versus autologous cells

Chondrocytes express transplantation antigens and can theoretically participate in immunological reactions although the matrix produced by chondrocytes protects cells from rejection. Experiments have demonstrated that allogeneic growth plate chondrocyte implantation in cartilage defects of adult rabbits yields neocartilage; however, it is degenerated after a few weeks not only due to the humoral immune response but also because of cell-mediated cytotoxicity.¹⁶ When comparing the long-term follow-up of allografted chondrocytes with autograft cells, both treatments gave cartilage regeneration. In humans, however, autologous chondrocytes seem to be preferable so far since there may be a risk of disease transmission with allografted cells.

16.15 Articular chondrocytes versus other cells

Articular chondrocytes have already been determined by the cartilage lineage, but other cartilage sources could be considered, for example, nose, rib ear, and growth plate chondrocytes that are equally suited to the generation of autologous cartilage grafts for nonarticular reconstructive surgery, and their regeneration potential could be enhanced using a combination of growth factors.¹⁷

Within the mesenchymal tissue, hyaline cartilage chondrocytes are usually considered tissue restricted and without broader differentiation potential.¹⁸ Culture-expanded mesenchymal stem cells (MSCs) have multipotency, and this gives rise to bone, cartilage, muscle, fat, and stromal cells under defined culture conditions. Hyaline chondrocytes are generally considered to lack multipotency and be restricted to cartilage lineage. However, when human articular chondrocytes are subjected to the same culture techniques and analysis used in the study of phenotypic plasticity of marrow-derived MSCs, chondrocytes have the potential to form cartilage in pellet mass cultures, adipose cells in dense monolayer cultures, and a calcium-rich matrix in an osteogenic assay. In contrast to MSCs, chondrocytes formed only cartilage, and not bone, in an *in vivo* osteochondrogenic ceramic implantation assay¹⁹; this indicates a different *in vivo* differentiation pathway. However, additional work will be necessary to assess whether cells of nonarticular origin can be successfully used for articular cartilage repair.

16.16 Embryonic stem cells and induced pluripotent stem cells

In the early 1980s, several laboratories were able to culture cells derived from the inner cell mass of 3.5 day-old mouse embryos. If cells are cultured on feeders or in conditioned media containing inhibitors of differentiation, embryonic stem (ES) cells are able to maintain their undifferentiated stage in cell cultures over numerous cell doublings. Since then, ES cells have been derived from several species including the human blastocyst. ES cells exhibit all the characteristics of stem cells with properties including self-renewal, pluripotency in differentiation, high levels of telomerase activity, short G1 cycle checkpoint, and ability to initiate DNA replication without external stimulation. When injected into muscles or testis of severe combined immunodeficiency (SCID) mice, the cells form teratomas with cells representing all three germ cell layers.

Embryonic stem cells cultured in confluent cultures that are devoid of their factors of inhibiting differentiation (leukemia inhibitory factor or continuous expansion of cells) demonstrate aggregate

formation. After several days in vitro, the cells form large aggregates or embryoid bodies (EBs) and when subjected to differentiation factors, the differentiation fate can be modified. Bone morphogenetic protein (BMP)-2 and -4 have been demonstrated to induce differentiation of chondrogenesis in mouse ES cell. In human ES cells, induced differentiation to the hyaline cartilage phenotype has been described if cocultured with irradiated human chondrocytes or subjected to a strict culture protocol.

The Nobel Prize in Physiology or Medicine 2012 was awarded jointly to Sir John B. Gurdon and Shinya Yamanaka “for the discovery that mature cells can be reprogrammed to become pluripotent.” The discovery that a limited number of genes (Oct4, Sox2, Klf4, and c-Myc) are able to reprogram adult human cells came as a revolutionary discovery that will have a broad implication on future cell therapies and tissue engineering efforts. Since then, numerous papers have been published in the field, but the use of retroviral vectors cause a change to the **induced pluripotent stem (IPS) cell** genome and make them unsuitable for cell therapy purpose. However, mRNA transfections with reprogramming factors are able to generate a footprint-free IPS cells that could potentially be used for cell therapy. The technology was recently applied in human chondrocytes where an IPS cell line was derived with a chondrogenic differentiation potential.

16.17 Xenograft cells

Another alternative cell source is **xenograft** cartilage, although the immunologic problem of cross-species transplantation, as well as the problem of cross-species pathogen infectivity, has to be solved if those cell types are to be used instead of true committed chondrocytes.²⁰

16.18 Direct isolation of tissue

Cell isolation and direct implantation have been described. The method involves a tissue fragmentation step to mince the cartilage tissue mechanically into small tissue fragments before reimplantation. The purpose is to promote outgrowth of embedded chondrocytes through the increased tissue surface area. Using this methodology, researchers established that chondrocytes can effectively grow into adjacently placed scaffold materials and produce neocartilage.²¹ The technique has also been successfully demonstrated in a rabbit model.²² If one uses such a technique, it might be possible to engineer cartilage by a one-stage procedure, in the future.

16.19 Scaffolds in cartilage tissue engineering

The primary goal of the tissue engineering approach to full-thickness cartilage defects is to provide engineered cartilage with the emphasis on **regeneration** rather than on repair. The ideal scaffold should provide an immediate support to cells and have mechanical properties matching those of the tissue being repaired. Gradually, the material then has to degrade, as the cells begin secreting their own ECM, and this thus allows for an optimal integration between newly formed and existing tissue.²³ (Table 16.2).

Cartilage repair with **allogeneic** or **autologous** cells of mesenchymal origin with different types of scaffolds has been tried in different models (Table 16.3). The successful molding of a human ear in PGA

Table 16.2 Criteria for scaffolds use in tissue engineering.

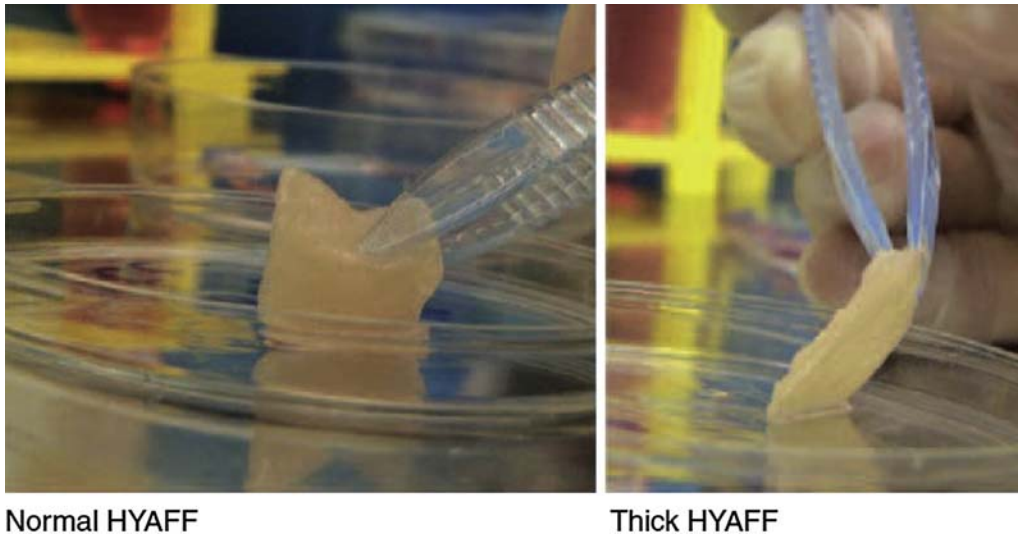
A	The surface should permit cell adhesion and growth.
B	Neither the polymer nor its degradation should provoke inflammation or toxicity when implanted in vivo.
C	The material should be reproducible in 3D structures.
D	The porosity should be at least 90% to provide a high surface area for cell–polymer interactions and sufficient space for ECM regeneration; minimal diffusional constraints during in vitro cultures.
E	The scaffolds should be absorbed once they have served the template function.
F	The scaffold degradation should match the rate of tissue regeneration.

Table 16.3 Different scaffolding materials used for cartilage repair.

Material	Absorbable fabricated matrices	References
Absorbable fabricated matrices		
Polygalactin	Vicryl, multifilament yarn	
Poly-L-lactic acid		
PGA	Dexon	Freed et al. ²³
Polyurethane		
Nonabsorbable fabricated matrices		
Polyvinyl alcohol	Ivalon, porous sponge	
Nylon		
Polyester	Dacron	
Polytetrafluoroethylene	Teflon	
Carbon fiber meshworks		
Polyethylene		
Matrices from animals or humans		
Collagen sponges (mainly type I collagen)		
Meniscus		
Decalcified bone		
Fibrin polymers		
Hyaluronic acid		

fibers subsequently seeded with calf articular chondrocytes and subcutaneously implanted in SCID mice demonstrated the potential of combination of artificial scaffolds and cells.²⁴

The use of a matrix for chondrocyte grafting started in the 1980s when embryonic chondrocytes in fibrin gel were used to maintain transferred chondrocytes within prefabricated cartilage defects in roosters.²⁵ Biodegradable PGA polymer seeded with chondrocytes was an alternative material used to resurface joint cartilage defects in the rabbit knee.²⁶ Type I collagen gel seeded with adherent cells

**FIGURE 16.9**

Cartilage graft construct based on chondrocytes cultured in hyaluronic acid.

from bone marrow or cells from the **periosteum** grown in culture were both able to regenerate cartilaginous tissue, although with mechanical properties similar to those of hyaline cartilage.²⁷ Despite numerous experiments, the combinations of scaffolds and cells have shown variable results, and the clinical use of these scaffolds has been lacking. In the search for the ideal scaffold material, hyaluronic acid has proven to be an ideal molecule for tissue engineering strategies in cartilage repair, given its impressive multifunctional activity in cartilage homeostasis.²⁸ (Fig. 16.9).

The recapitulation of embryonic events represents a major goal for tissue engineering, but it is important to emphasize that the adult environment is quite different from the one present during the embryonic stage and that tissues respond differently to specific information.²⁹

All this is performed through complicated multistep differentiation cascades that imply fine cross-talk between the chondrocytes and ECM with the synthesis of site-specific specialized molecules. Therefore, the appropriate scaffold should be designed to play this multifunctional role and not be used as a mere vehicle for reparative cells or growth factors. The scaffold has to change its features as the regenerative process takes place—starting with tissue induction in the early events and then supporting tissue formation during differentiation. With this notion in mind, the materials used in tissue engineering have to be biocompatible and biodegradable as well as, promote cell attachment, and ultimately possess the capability of being remodeled by tissue-specific cells.

Hyaluronan is present in all soft tissues of higher organisms and, in particularly high concentrations, in the ECM of articular cartilage and in the mesenchyme of the developing embryo.³⁰ It is a linear polymer consisting of a regular repeating sequence of a nonsulfated disaccharide units, glucuronic acid, and N-acetyl-glucosamine with a molecular weight varying from 4000 to 8×10^6 Da. Hyaluronan is basically unmodified by evolution, which underlies its importance in the physiological environment. Hyaluronan plays different biological roles depending on its physical and chemical properties. It interacts with binding proteins, proteoglycans, and active molecules, such as growth factors that influence matrix

structure, water balance lubrication, and cell interaction. Hyaluronan can influence cell movement by its ability to alter the osmotic pressure leading to the formation of hydrated pathways in the tissue.

The use of hyaluronan, in its highly purified form, has become common practice in medicine. Intra-articular administration of hyaluronan is widely being used to relieve pain and improve joint mobility in the nonsurgical treatment of OA. Hyaluronan is a major component of the ECM of embryonic mesenchymal tissues; thus, the use of hyaluronan-based scaffolds will help create an embryonic-like milieu.

The greatest barrier to the successful use of hyaluronan in tissue engineering is water solubility, rapid resorption, and short residence time in the tissue along with its poor ductility. Cross-linking and coupling reactions are two ways to obtain a modified, stable form of hyaluronan.³¹ With the right application, hyaluronan derivatives can be extruded to produce fibers or membranes, lyophilized to obtain sponges, or processed to obtain microspheres. The combination of hyaluronan-based scaffolds and fibers has found its way into the clinic, where several thousand patients have now been treated.^{32,33}

16.20 Bioreactors in cartilage tissue engineering

Despite the good results of the first and second generation technology of ACI, some of the concerns regarding the transplantation of immature chondrocytes in suspension led to the third generation of ACI with either cells grown on a cell carrier-like matrix-assisted chondrocyte implantation or involving a three-dimensional (3D) scaffold for cells to grow into in a porous scaffold like Hyalograft. After cell expansion and cell culture, the whole cell-seeded scaffold is implanted into the defect. This technique has several potential advantages such as the possibility of arthroscopic implantation, predifferentiation of cells in vitro, and implant stability. The nutritional requirements of cells that synthesize ECM increase along the differentiation process. Bioreactors therefore represent a great tool to accelerate the biochemical and mechanical properties of the engineered tissues and provide adequate mass transfer and physical stimuli. Such bioreactor model systems have been created based on chondrocytes and biodegradable PGA scaffolds, where the scaffold induces cell differentiation and degrades at a defined rate, whereas the bioreactor maintains controlled in vitro culture conditions that permit tissue growth and development. The bioreactor has enabled tests for cartilage formation in different gravitations where Earth gravitation was superior to low gravitation in space.³⁴

16.21 Growth factors that stimulate chondrogenesis

The cartilage matrix contains several growth factors that are released at the time of injury from the damaged cartilage matrix as well as from the damaged subchondral bone. Such growth factors include transforming growth factor- β s (TGF β), insulin-like growth factor-I (IGF-I), BMPs, and basic fibroblast growth factor (FGF-2).³⁵ The factors orchestrate different effects. FGF2 stimulates chondrocyte cell proliferation, stabilizes phenotypic expression, and inhibits terminal chondrocyte differentiation. TGF- β controls chondrocyte proliferation and differentiation and induces chondrogenic differentiation of mesenchymal cells in vitro and chondrogenesis in vivo.³⁶ It also inhibits chondrocyte **terminal differentiation** and stimulates chondrocyte proliferation in cooperation with FGF2, and it has been shown to promote the repair of damaged cartilage. BMPs initiate cartilage differentiation and maintain the chondrocytic phenotype. IGFs have important anabolic effects on cartilage metabolism with increased

matrix production. These findings indicate a potential therapeutic way to use exogenous growth factors to enhance the repair of cartilage defects.

16.22 Future developments in cartilage biology

Research in regenerative medicine involves cell and molecular biology, developmental cell biology, immunology, and polymer chemistry. This new direction in medicine will use three strategies: transplantation of cells to form new tissue in the transplant site, implantation of bioartificial tissues constructed in vitro, and induction of regeneration in vivo from healthy tissues next to an injury.³⁷

Recently, different fourth generation ACI techniques have been presented. One stage procedure with direct isolation of chondrocytes or chondrons is mixed with either autologous or allogeneic MSCs.

The use of particulated or fragmented autologous or allogeneic cartilage as a source for chondrocytes is also regarded as a fourth generation ACI. From crushed cartilage, the most active chondrocytes may migrate out into a surrounding supportive scaffold, gel, or similar.

However, regarding the future for cartilage repair, the idea is to transplant stem/progenitor cells, or their differentiated products, into a cartilage lesion site where they may form new tissue, or the cells could be used to construct a bioartificial tissue in vitro to replace the original tissue or organ. Bioartificial tissues are made by seeding stem cells or differentiated cells into a natural or artificial biomaterial scaffold shaped in the appropriate form and then by implanting the construct in place of the damaged tissue or organ. Theoretically, the use of cartilage stem cells is preferable to the use of differentiated cells harvested directly from a donor because stem cells have the potential for unlimited growth and thus supply. Such so-called uncommitted cells are capable of a broad range of chondrogenic expression and could provide a regenerative tissue that recreates the embryonic lineage transitions originally involved in joint tissue formation. The technology of IPS cells could also be a potential source of a universal donor tissue cell line. The use of 3D printing technology provides a possibility of printing chondrocytes in matrix with exact fit into the cartilage defect. However, the recent research described in this chapter shows that the use of true committed chondrocytes is still the most reasonable methodology.

16.23 Introduction: bone—basic bone biology: structure, function, and cells

Bone comprises the largest proportion of the body's connective tissue and serves a number of key functions including (1) structural support, (2) protection of internal organs, (3) locomotion, and (4) maintenance of mineral balance, and as a reservoir for growth factors and for hematopoiesis.³⁸ Bone is unique as it has the capacity to renew and remodel throughout life. Bone can be separated into two main types: **cortical** (compact) and **cancellous** (trabecular or spongy) **bone**. 80%–90% percent of cortical bone is calcified and is located at the diaphysis of long bones, on the surface of flat bones and as a thin shell (Fig. 16.10) around the epiphyses and metaphysis of long bones to provide mechanical and protective functions. In contrast, only 15%–25% of cancellous or trabecular bone is calcified. Cancellous bone, typically a 3D lattice that comprises bone plates and columns, forms the epiphyseal and metaphyseal regions of long bones and the cortex of the flat bones and provides the metabolic

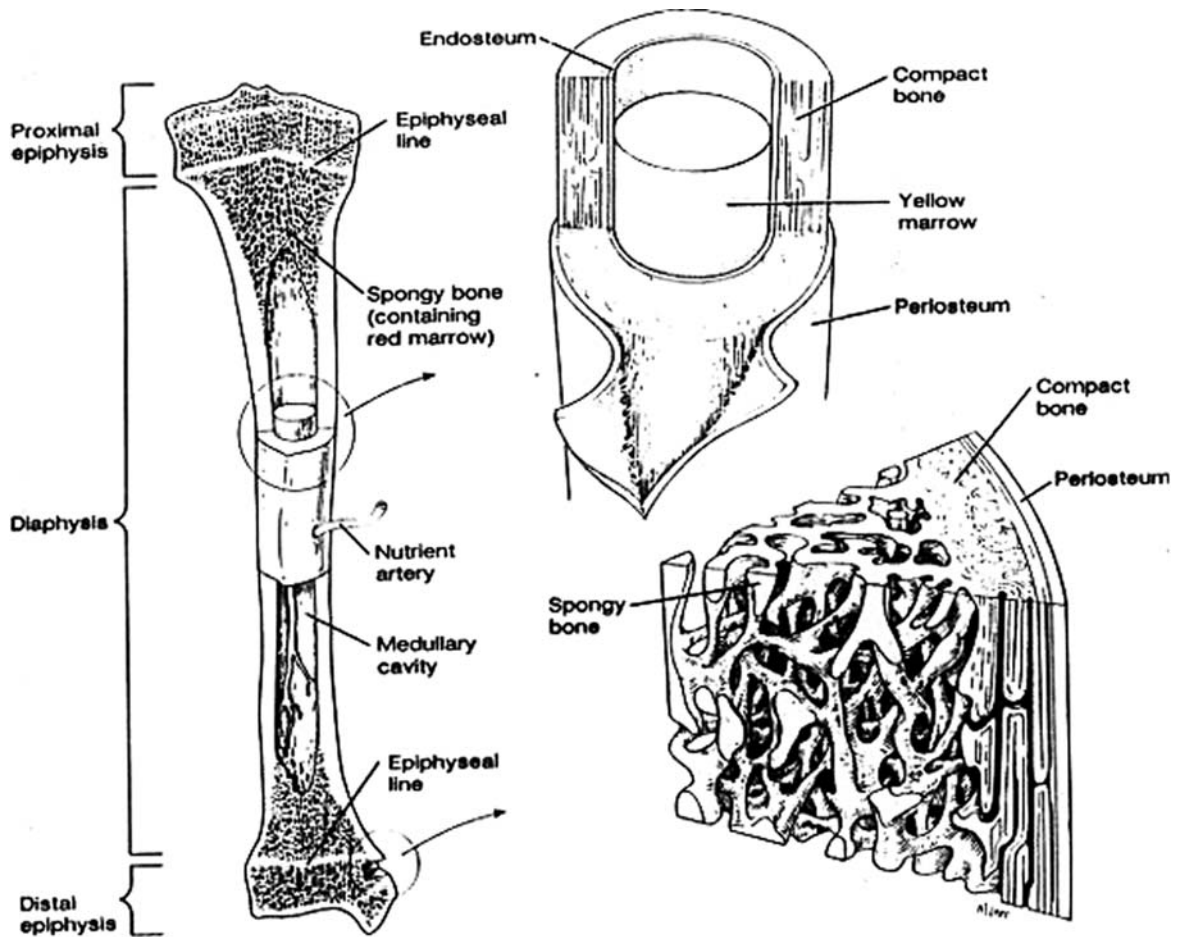
**FIGURE 16.10**

Diagram of the structure of a long bone. From *Basic Human Anatomy*, third ed., Spence A, Addison—Wesley, Redwood City, CA; 1991.

functions in bone.³⁹ The ECM of bone consists of mineral, collagen, water, noncollagenous proteins, and lipids. The mineral phase is predominantly hydroxyapatite $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$, with small quantities of carbonate, magnesium, and acid phosphate. This provides bone with its characteristic mechanical rigidity and compressive strength. In contrast, the organic matrix is mostly composed of type I collagen with small amounts of type III, V, and nonfibrillar collagens, with approximately 10%–15% of the organic matrix comprising noncollagenous proteins. Noncollagenous proteins include albumin, α_2 -HS-glycoprotein, and growth factors and noncollagenous proteins synthesized by osteoblasts (proteoglycans, glycosylated proteins, glycosylated proteins, and γ -carboxylated proteins) (Table 16.4). The organic components of bone provide bone with elasticity and flexibility.³⁸

Table 16.4 Extracellular matrix proteins in bone.

Protein		Function/Location	Disease (absence or dysfunction)
Collagen-related proteins	Type I	Binds and orientates other proteins	Osteogenesis imperfecta
	Type X	Present in hypertrophic cartilage	Metaphyseal chondroplasia
	Type III	Trace, may regulate collagen fibril diameter	Ehlers—Danlos syndrome
	Type V	Trace, may regulate collagen fibril diameter	Disrupted fibrils in mouse model
Serum proteins	Albumin	Inhibits HA crystal growth	None
	AHSG	Promotes endocytosis Monocyte chemoattractant Inhibits calcification	Ectopic calcification in mouse knockout
GAG-containing molecules	Aggrecan	Matrix organization and strength Retention of H ₂ O, Ca, PO ₄	Spondyloepiphyseal dysplasia
	Versican	Regulates chondrogenesis Defines space destined to ossify	Wagner syndrome
	Decorin	May regulate collagen fibril size May modulate TGF- β activity	Progeroid form of Ehlers—Danlos syndrome (with double decorin—biglycan knockout)
	Biglycan	Determines peak bone mass May regulate growth factor activity	
	Asporin	Regulates collagen structure	Human polymorphism associated with OA
Glycoproteins	Hyaluronan	May define space destined to ossify	None
	Alkaline phosphatase	Hydrolyzes inhibitors of mineral deposition Increases local PO ₄ concentration	Hypophosphatasia—poor growth and mineralization
	Osteonectin	Regulates collagen organization May regulate HA deposition Regulates growth factors	Low mineral content Osteopenia in knockout mouse
SIBLING proteins	Osteopontin	Binds to cells, controls proliferation May regulate mineralization Inhibits nitric oxide synthase	Poor osteoclast remodeling Increased mineral content in knockout mouse
	Bone sialoprotein	Binds to cells May initiate mineralization	None
	MEPE	Regulation of PO ₄ metabolism	Oncogenic osteomalacia

Continued

Table 16.4 Extracellular matrix proteins in bone. *Continued*

Protein		Function/Location	Disease (absence or dysfunction)
RGD-containing glycoproteins	Thrombospondins	Cell attachment Binds to collagens, platelets, thrombin, fibrinogen, laminin, plasminogen, and PAI	Pseudoachondroplasia
	Fibronectin	Cell attachment Binds to fibrin, heparin, gelatin, collagens	Lethal before skeletal development in knockout mouse
	Vitronectin	Cell attachment Binds: collagens, plasminogen, PAI, heparin	None
	Fibrillin 1 and 2	Regulates elastic fiber formation	Marfan syndrome in Fibrillin 1 mutation
	Matrix Gla protein	Inhibitor of mineralization	Keutel syndrome (excessive cartilage mineralization)
	Osteocalcin	Regulates osteoclasts Inhibits mineralization May coordinate bone turnover	Knockout mouse—osteopetrosis
	Protein S	May be synthesized by osteoblasts	Osteopenia
γ -Carboxy glutamic acid-containing proteins			

AHSG, α 2HS glycoprotein; Ca, calcium; HA, Hydroxyapatite; H₂O, water; MEPE, Matrix extracellular phosphoglycoprotein; OA, Osteoarthritis; PAI, Plasminogen Activator Inhibitor; PO₄, phosphate; RGD, Arginyl-glycyl-aspartic acid DNA motif; SIBLING, Small Integrin Binding Ligand N-Glycosylated; TGF- β , Transforming Growth Factor Beta.

Adapted from Clarke. 38.

The structure of a long bone (femur) (Fig. 16.10) can be separated into distinct regions. The central shaft of a long bone is called the diaphysis, and it has a hollow center, the medullary cavity, containing the bone marrow. In children, long bones are filled with red marrow that is gradually replaced with yellow marrow with age. The diaphysis is predominantly composed of compact bone with only a small component of spongy bone on its inner surface around the medullary cavity. The end zone of the diaphysis is referred to as the metaphysis; and at each end of the long bone is located the epiphysis (proximal/distal). The metaphysis is the developmental zone between the epiphysis and the diaphysis, known as the “growth plate” or “epiphyseal plate.” The growth plate is central in the process of endochondral ossification and enables growth during childhood. The joint surface of each epiphysis is covered by a thin layer of articular cartilage that protects the opposing bone ends during joint movement and serves to also absorb stress.⁴⁰ The articular cartilage covers a layer of compact bone, beneath which trabecular bone resides.

Cortical bone is made up of an interconnected group of subunits known as **osteons**, or the Haversian system (Fig. 16.11). A single osteon consists of a hollow, laminated rod of collagen, and inorganic calcium phosphates (CPs) with a hollow core, the Haversian canal that contains a blood and nerve vessel. Surrounding the outer surface of most bones, except at the joints, is the periosteum. The periosteum contains a double-layered membrane—an outer fibrous layer of collagen fibers and an inner layer of skeletal cells including skeletal stem cells (SSCs). The periosteum has a number of blood and lymphatic vessels, as well as nerve fibers, and serves to provide much of the bone nutrients as well as a key role in appositional bone growth and fracture repair.

The periosteum also gives rise to important muscular tendon and ligament insertions key in facilitating locomotion. The endosteum lines the inner bone surfaces and is composed of a single layer of connective tissue that lines the surface of the medullary cavity.

Flat bones are found where the principal requirement is either protection or provision of broad surfaces for muscular attachment. These bones are typically, broad, flat plates (e.g., cranium, ilium, and scapula)

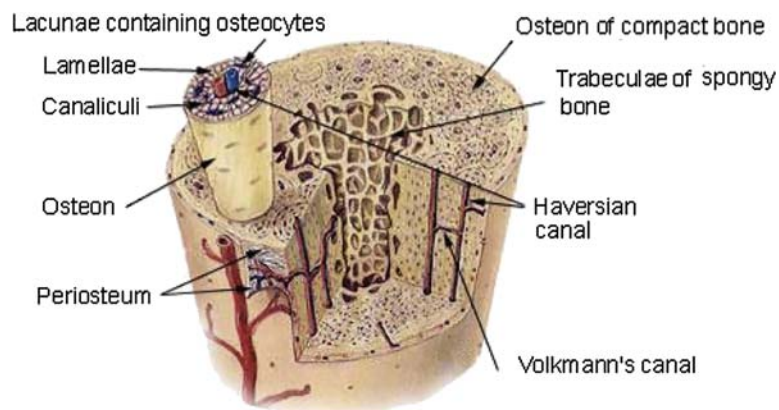


FIGURE 16.11

The microstructure of bone. From *The Human Bone Manual*, White and Folkens, Academic Press; 2005.

and comprise two thin plates of cortical bone with cancellous bone between the plates. Similarly, short bones, such as the wrist, and irregular bones, such as the spine and hip, also consist of cortical bone surrounding trabecular bone.³⁹

Osteoblasts are responsible for the synthesis and secretion of hydroxyapatite and proteins which form the ECM of bone. Formation of osteoblasts is governed by the interaction of stimulatory factors TGF- β , BMP-2, 4, and 7, and the presence of their inhibitor's noggin, chordin, gremlin, and sclerostin. Osteoblasts may become encased in matrix and become **osteocytes** which are pivotal in transducing the effect of mechanical stimulation on bone formation. Bone breakdown observed in both remodeling and pathological conditions is mediated by **osteoclasts** which are derived from myeloid precursor cells. Upon stimulation, osteoclasts form a ruffled membrane and secrete acid to dissolve hydroxyapatite, subsequently proteolytic enzymes breakdown the organic matrix. In addition to local mechanical stimulation, the metabolic activity of bone is also determined by calcitonin and parathyroid hormones which mediate serum calcium homeostasis.

16.24 Intramembranous and endochondral bone formation

The de novo formation of trabecular and cortical bone involves the differentiation of skeletal cells into osteoblasts that synthesize osteoid (nonmineralized bone matrix) and subsequently mineralize the matrix. It is important to note that skeletal cells need a substrate upon which the bone will be deposited, either a membrane (as in intramembranous ossification), preexisting cartilage spicules (as in endochondral ossification), or preexisting bone.⁴¹ The flat bones of the skull, a number of facial bones, and most of the clavicle form by **intramembranous ossification**. Intramembranous ossification is a process in which bone is laid down by direct apposition within stromal connective tissue. In contrast, long bones develop predominantly by **endochondral ossification**⁴² in which mesenchymal cells cluster to form a cartilage template or "anlage," which acts as a scaffold on which the new bone is formed. Intramembranous and endochondral ossification are described in greater detail in [Chapter 3 Tissue formation during embryogenesis](#).

16.25 Fracture repair

Fractures in which bone fragments are anatomically reduced and rigidly held may heal via a process known as primary bone healing. Typically, some degree of interfragmentary motion is present in which case bone healing occurs via callus formation, termed secondary healing.

Primary fracture healing occurs only when strain $<2\%$ ⁴³ and is usually only observed following open reduction and rigid internal fixation. Primary healing may be subcategorized into contact healing and gap healing in which the distance between fragments is <0.01 mm and <1 mm, respectively.⁴⁴ During contact healing, osteoclasts form tips of cutting cones which cross the fracture site creating longitudinal cavities which are filled with bone produced by osteoblasts. This process simultaneously results in bony union with axially orientated Haversian systems. Gap healing differs in this regard as the initial lamellae formed are perpendicular to the longitudinal axis and are subsequently remodeled to restore the Haversian system and mechanical properties.

Secondary fracture healing occurs following treatment in a cast, or with less rigid forms of fixation such as intramedullary nailing and involves both endochondral and intramembranous ossification. While micromotion stimulates secondary fracture healing, too much motion results in hypertrophic nonunion, which can be remedied with more rigid surgical fixation.

Secondary bone healing can be conceptualized into four stages: acute inflammation with hematoma formation, soft callus formation, hard callus formation, and finally remodeling, which is analogous to primary healing.⁴⁵

By definition, the initial fracture results in disruption of bony architecture and surrounding soft tissue, with accompanying loss of mechanical stability and vasculature. A fibrin rich clot forms at the fracture which is reorganized into granulation tissue under the exquisite control of cytokines and growth factors (including TGF- β , platelet-derived growth factor (PDGF), basic FGF-2, vascular endothelial growth factor (VEGF), macrophage colony-stimulating factor, BMPs, interleukin (IL)-1 and IL-6, and tumor necrosis factor-alpha (TNF- α)) produced by the infiltrating cells. These factors are responsible for the recruitment and stimulation of stem cells from bone marrow, periosteum, blood vessels, and surrounding soft tissue. Nonsteroidal antiinflammatory drugs which inhibit cytokine production via inhibition of cyclooxygenase are known to delay fracture healing. Removal of devitalized tissue via neutrophils and macrophages also is crucial, as remaining necrotic material promotes chronic inflammation leading to nonunion.

In the second phase of fracture healing, endochondral ossification commences via soft fibrocartilaginous callus formation at the fracture ends and external to the periosteum. Granulation tissue is replaced by fibrocartilage synthesized by chondrocytes and fibroblasts and eventually forms a semirigid cartilaginous template that holds the fracture. Intramembranous ossification also commences subperiosteally during this period.

Hard callus formation is subsequently driven by proangiogenic factors, including VEGF, BMPs, FGF-1, and TGF- β , which stimulate callus vascularization, chondrocyte hypertrophy, and apoptosis. SSCs from the periosteum, bone marrow, vasculature, and surrounding soft tissues aid hard callus formation as bone progenitors differentiate under the actions of factors, such as the BMPs, to form osteoblasts and the generation of mineralized bone matrix resulting in the formation of woven bone.

During remodeling, anisotropic woven bone undergoes resorption by osteoclasts, and lamellar bone is deposited by osteoblasts thereby restoring bone architecture according to the biomechanical environment. While remodeling may commence within a month, the process can take years to complete, with adequate vascularity and a gradual increase in mechanical stability vital for completion.

16.26 Critical size defect

Within research and clinical practice, the term **critical size defect** lacks a precise definition but tends to refer to a bone defect which would not heal spontaneously following surgical stabilization without an additional procedure such as autologous bone grafting.⁴⁶ Critical size defects lack consistency as in addition to size; anatomical location of the defect, state of surrounding soft tissues including vasculature, and systemic factors such as smoking are critical in determining healing potential. Considerable

variation in bone defect healing is also observed with both age and species. It is noteworthy that a critical size defect is not analogous to fracture nonunion. In some cases, researchers may have selected a critical size bone defect as a surrogate *in vivo* model for fracture healing as bone defects can be produced intraoperatively with greater consistency than fractures. Generally, fracture nonunion results from inadequate biological stimulation (atrophic nonunion) or inadequate stability (hypertrophic nonunion). A critical size defect may benefit from favorable biology and adequate stability but merely be unable to regenerate the volume of bone lost.⁴⁶

16.27 Skeletal stem cells

Bone marrow stromal tissue contains a subset of nonhematopoietic progenitor cells that exhibit the two characteristic features of stem cells, namely, the capacity for prolonged or unlimited self-renewal under controlled conditions and the potential to differentiate into a variety of mature cell types. The multipotent, self-renewing progenitor cells of bone marrow stroma are widely referred to as MSCs, a term originally coined by Caplan⁴⁷ and extensively used in the literature, notably by Pittenger et al.⁴⁸ (see [Chapter 2 Stem cells](#)). The term “skeletal stem cells” was introduced by Bianco and Robey to denote specifically adult stem cells derived from bone marrow stromal tissue (see, for example, Bianco and Robey)⁴⁹ with the ability to generate bone, cartilage, and stromal tissue upon reimplantation *in vivo*. This concept builds on early work by Friedenstein and coworkers who first confirmed the presence of fibroblast-like cells that form surface adherent colonies in tissue culture (colony-forming unit-fibroblastic/CFU-F) and form bone ossicles when retransplanted *in vivo*.⁵⁰

An array of cell surface markers have been explored to prospectively isolate stem cells with *in vivo* bone forming potential (see [Chapter 2 Stem cells](#)); however, the identity of a single unique cell surface marker that specifically recognizes SSCs still remains elusive. In the field of skeletal science, there are two distinct perspectives regarding the biological nature and function of SSCs. Bianco et al. are of the view that the SSC is a cell with a “precisely defined physical and conceptual entity” and it cannot be described outside of its *in situ* context. This is based on the results of their seminal work, which provided compelling evidence that the SSCs reside in the perivascular spaces surrounding the vascular sinusoids in the bone marrow in a subendothelial position.⁵¹ Perisinusoidal SSCs were found to express the STRO-1 antigen, CD146, CD105, alkaline phosphatase, and VCAM-1, and their transcriptome was constituted by genes distinctive of mural cells (i.e., vascular smooth muscle cells and pericytes) and committed osteoprogenitor cells, as well as those related to the HSC niche, but was devoid of endothelial and late osteogenic genes.⁵¹ Thus, in accordance with the perspective of Bianco et al., upon transplantation *in vivo*, an SSC is a single cell capable of generating a complete heterotopic bone or bone marrow organ/ossicle (including a compartment of perivascular stromal cells with similar phenotypes and properties as those of the originally explanted cell) in which hematopoiesis from the recipient animal becomes established ([Fig. 16.12](#)). The tissue culture plastic–adherent nonhematopoietic fraction of bone marrow is heterogeneous as it contains both osteoprogenitors and SSCs ([Fig. 16.12a](#)). Upon ectopic implantation with a suitable osteoinductive carrier in immunocompromised mice, osteoprogenitor cells are only able to form bone, while relatively homogenous populations of SSCs, immunoselected on the basis of expression of one or more SSC-specific surface markers, are able to regenerate bone and stroma and, critically, establish a hematopoietic microenvironment and, critically, establish

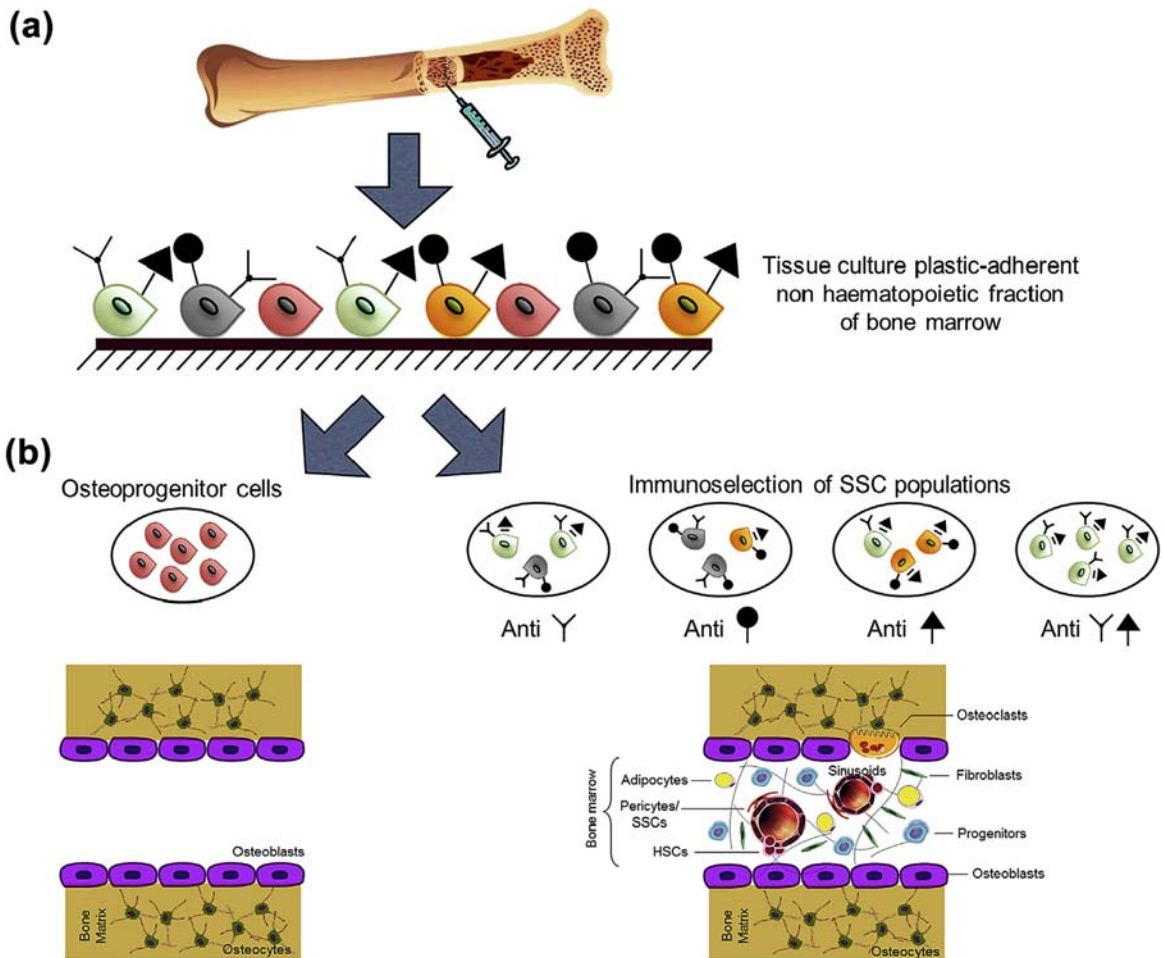


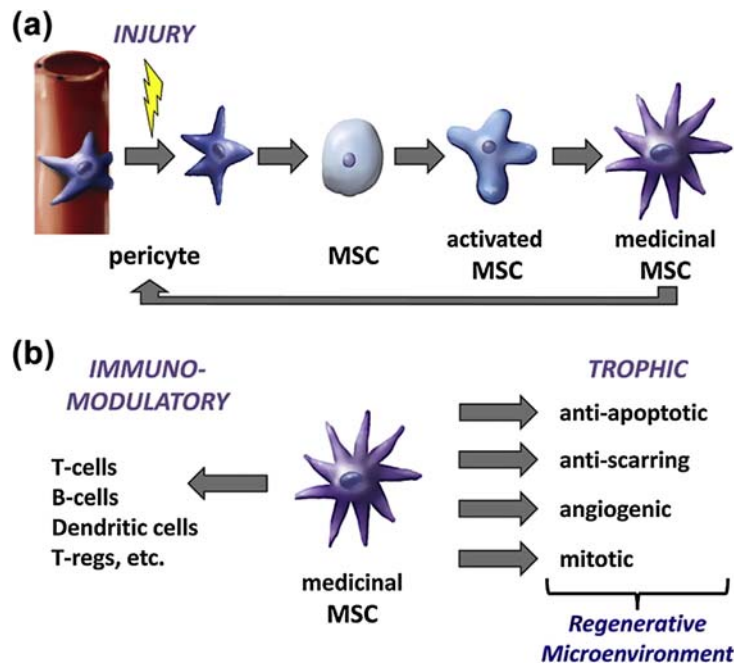
FIGURE 16.12

SSCs establish, organize, and transfer the hematopoietic niche in vivo. From Dawson JI, Kanczler J, Tare R, Kassem M, Oreffo RO. *Concise review: bridging the gap: bone regeneration using skeletal stem cell-based strategies—where are we now?* Stem Cells. 2014;32(1): 35–44.

a hematopoietic microenvironment. The capacity to establish, organize, and transfer the hematopoietic niche in vivo is regarded as an important defining feature of SSCs (Fig. 16.12b).

In contrast, Caplan and Pittenger are of the view that MSCs are a product of their environment that can exist or be developed in other locations, including the culture dish, that is, in vitro.^{47,48,52} In accordance with the perspective of Caplan and Correa,⁵² MSCs exhibit the following two phenotypes:

1. a “constitutive” phenotype in which, as perivascular cells, the population expresses the characteristic cell surface markers both in vivo and ex vivo and demonstrates functionality through its multipotential ex vivo differentiation capabilities.
2. a “regulatory” phenotype in situations of tissue/vessel damage in vivo, where the released pericytes are activated by injury to become MSCs, which secrete a variety of bioactive molecules as part of their local trophic and immunomodulatory activities that help to establish a regenerative microenvironment to support the repair of the injured tissue (Fig. 16.13). The sequential activation of pericytes as a response to injury is explained. Local vessel damage affects resident pericytes and liberates them from functional contact with blood vessels to become activated MSCs. Upon immune activation, these mobilized, “medicinal” MSCs secrete factors that organize a regenerative microenvironment. Subsequent repair is reinforced when activated MSCs reacquire a stabilizing pericyte phenotype in the abluminal space (Fig. 16.13a). The bioactive molecules secreted by medicinal MSCs are immunomodulatory and affect a variety of immune cell lineages. Other secreted molecules establish a regenerative microenvironment by establishing a powerful trophic field (Fig. 16.13b). In this context, MSCs serve as site-regulated, multidrug dispensaries to promote and support the natural regeneration of focal injuries. The natural supply of MSCs needs to be supplemented by local or systemic delivery in situations where the injuries are large or occur in older individuals.

**FIGURE 16.13**

MSCs are immunomodulatory and trophic. From *The MSC: an injury drugstore*, Caplan and Correa, Cell Stem Cell. 2011;9:11–15.

16.27.1 Immunomodulatory properties

In addition to their stem/progenitor properties, there is considerable interest in the immunomodulatory properties of SSCs and a large body of literature has documented the pleotropic effects of SSCs on cells of the immune system⁵² (see [Chapters 2 Stem cells](#) and [Chapter 12 Controlled release strategies in tissue engineering](#)). Macrophages are key players of the innate immune system in initiating and controlling inflammation, and SSCs can influence macrophage function depending upon the inflammatory context. Monocytes arriving at an inflammatory environment can develop into activated macrophages, which produce proinflammatory cytokines such as TNF- α and IFN- γ . The resulting proinflammatory microenvironment activates SSCs to adopt an immune-suppressive phenotype and release mediators, which skew the differentiation of monocytes toward antiinflammatory macrophages that secrete high levels of IL-10, TGF- β 1 (supports SSC-mediated generation of regulatory T-cells/Tregs that suppress T-cell-mediated immune responses), and low levels of TNF- α , IFN- γ , IL-1, IL-6, and aid tissue regeneration following inflammation (see [Chapter 2 Stem cells](#)). While, in the context of Tissue Engineering, the regenerative tissue forming properties of stem cells are typically emphasized, the potential of immune-modulatory influence by stem cells is increasingly recognized as having a key role in promoting the successful engraftment and function of implanted tissue engineered constructs.⁵³

16.28 Expansion and differentiation

SSCs are characterized by limited capacity for proliferation in monolayer cultures and typically exhibit “replicative senescence” as a consequence of repeated subculture. The problem is exacerbated further in SSCs from elderly individuals due to accelerated senescence following ex vivo culture. Development of simple, safe, and efficacious culture techniques that allow ex vivo expansion of SSCs without loss of their functional, immunophenotypic, and cytogenetic characteristics (see [Chapter 12 Controlled release strategies in tissue engineering](#)) is therefore one of the key requirements for the successful application of SSCs in bone regeneration.

16.29 Growth factors for bone repair

Bone formation involves the complex interplay of a number of hormones and growth factors. The targeted delivery of growth factors to recruit and promote osteogenesis of transplanted or endogenous stem cell populations represents an important strategy in bone tissue engineering and regenerative medicine.⁵⁴ The growth factors most extensively characterized for their ability to stimulate osteogenesis are the BMPs. Clinical interest has been centered particularly on BMP-2, BMP-4, and BMP-7 in the potential treatment of fracture repair, large segmental bone defects, spinal fusion, and in the fixation of prosthetic implants.⁵⁵ An ongoing challenge for the clinical application of BMPs is the supraphysiological doses typically required for efficacy and concerns over immunogenicity and the risk of ectopic bone formation associated with high dosage.

Other important local signaling molecules of relevance to bone regeneration include the TGF- β subfamily (TGF- β s), the Wnt proteins (Wnts), IGFs, FGFs, PDGFs, and the VEGFs (see [Chapter 4 Cell signaling](#)).

16.29.1 Scaffolds for bone regeneration

The scaffold provides an extracellular microenvironment for the support and stimulation of stem cell–driven tissue regeneration. It can host transplanted cells, recruit endogenous cells, mediate biological signaling, and provide mechanical support. Along with classic bone graft strategies (autologous and allogeneic bone graft), bone tissue engineering scaffolds can be composed of CP-based ceramics, extracted xenogeneic ECM components, and naturally or synthetically derived polymers. These various materials and forms possess intrinsic strengths and weaknesses (Table 16.5), and thus, there is an expanding body of research engaged in modifying and optimizing scaffold design to meet the complex of specifications required for clinically relevant bone regeneration (Table 16.6 and Fig. 16.14).⁵⁶ An example is hydroxyapatite scaffolds generated with a defined 100- μ m porosity via the additive manufacture (AM) technique extrusion free forming (Fig. 16.14a). Along with macropore control via AM, microporosity can be controlled via the temperature of sintering (Fig. 16.14b). In the porous decellularized bone graft, viable HBMSCs can be seeded (Fig 16.14; cells stained a green fluorescent dye). Injectable hydrogels that set in situ allow minimally invasive delivery of cells and growth factors (Fig 16.14d; see how the injectable hydrogel can instantly gel and connect two pieces of tissue).

Bioceramics incorporating hydroxyapatite or CPs are able to integrate well with bone tissue and have a high compressive strength. However, such ceramics do not in themselves stimulate bone formation (osteinductivity), and while increased porosity facilitates greater penetration of new bone formation, complete resorption of a ceramic scaffold material is rarely observed.

Natural polymers, particularly those derived from extracted ECM components, such as collagens, fibrin, or the GAG hyaluronic acid, have the inherent ability to modulate cell behavior via complex cell–matrix interactions. These biologic materials are intrinsically biocompatible, and while their complexity typically precludes mass synthesis and so require a xenogeneic source, biologic materials have been used for many years in humans with minimal adverse immunological outcomes.⁵⁷

Synthetic polymers have also been extensively developed as scaffold materials, due, not least, to their sheer versatility. The physical and chemical properties of these macromolecules can be modulated on the basis of their monomer constituents, the relative ratios of copolymers, the interactions of polymers with one another (i.e., in the mode and degree of cross-linkage), and the incorporation of side chains. In addition, side chains can be functionalized by the covalent immobilization of biologically active molecules, such as adhesion factors and cytokines that, together with hydrophilic molecules otherwise resistant to cell adhesion and protein absorption, provide a highly defined mode of controlling specific cell responses.⁵⁸

Hydrogels are hydrated polymer chains, while alone they have not been shown to enhance bone formation; they have been used effectively in the delivery of cells and growth factors. Naturally derived hydrogels extensively investigated as cell, growth factor, and drug delivery vehicles in bone regeneration include gelatin, hyaluronic acid, fibroin, collagen, and alginate. Polyethylene glycol and derivatives represent the most commonly used synthetic gels.

16.30 Scaffold biocompatibility

Perhaps the most basic property required of a bone tissue engineering scaffold is biocompatibility. Naturally occurring polymers and bioceramics tend to display good biocompatibility due to their

Table 16.5 Scaffold materials and their relative strengths and weaknesses for bone tissue engineering.

	Class of biomaterial								
	Autograft	Allograft	DBM	ECM scaffolds		Non-ECM-derived scaffolds			
				Fibrous scaffolds	Hydrogels	Solid-wall scaffolds		Fibrous polymer	Polymer hydrogel
						Polymer	Ceramic		
Manufacture/Clinical specifications									
Allow economical sourcing and manufacture	–	+	+	+	+	++	++	++	++
Manufacture according to GMP	NA	+	+	+	+	++	++	++	++
Meet FDA approval	NA	+	+	±	±	±	+	±	±
Amenable to sterilization	NA	-	-	-	-	++	++	++	++
Good handling/implantation properties	-	+	-	+	++	+	-	+	++
Nonimmunogenic	++	-	-	++	++	++	++	++	++
Controlled 3D architecture/geometry	-	-	-	+	++	++	±	++	++
Impart provisional weight bearing functionality	++	++	-	-	-	±	++	-	-
Radiologically distinguishable	+	+	-	-	-	-	+	-	-
Scaffolding specifications									
Biocompatible	++	+	+	+	+	+	+	+	+
Provide mechanical support for surrounding tissue	+	+	-	±	-	+	+	+	±
Facilitate cell delivery	++	-	-	+	++	+	+	+	++
Controlled degradation	++	++	-	+	+	+	+	+	+
Conducive for cell ingrowth/migration	++	+	+	++	±	+	+	++	±
ECM specifications									
Provision of cell–matrix interactions	++	+	+	+	+	-	+	-	-
Transmission of mechanical signals to cells	++	++	-	+	+	+	-	+	+
Localization of bioactive factors	++	-	+	-	-	-	+	-	+
Extended release of bioactive factors	++	-	+	-	-	-	+	-	±
Preserved bioactivity of bioactive factors	++	-	+	-	-	-	+	-	±

Key: ++ particular strength, + strength, ± variable between specific materials, - weakness, – particular weakness. Relative strengths and weaknesses are generalized characteristics commonly noted for the various materials. Scaffold modifications and the generation of composite scaffolds overcome many of these weaknesses (see Table 16.6).

Table 16.6 Modifications to scaffold design associated with enhanced bone formation in vivo.

Class of modification	Examples of modifications that have promoted bone formation
Porosity/pore size	Increased interconnection between pores Incorporation of macroscopic channels Increased porosity Higher surface area for bone apposition
Degradation/resorption rate	Controlled degradation Slower degradation if faster than bone remodeling Faster resorption if slower than bone remodeling
Bone mineral incorporation	Apatite coating Incorporation of calcium phosphate particles
ECM protein incorporation	Addition of demineralized bone matrix ECM protein surface coating ECM-based hydrogels
Surface properties/topography	Addition of nanotopographical features Modification of surface roughness Surface hydrophobicity/hydrophilicity
Bioactive factor incorporation	Controlled release of growth factors Growth factor surface binding Bioactive peptides/small molecule incorporation

Many additional modifications not listed above have shown improved bone formation in vitro or else offer enhancements not directly assayable by in vivo bone forming assays.

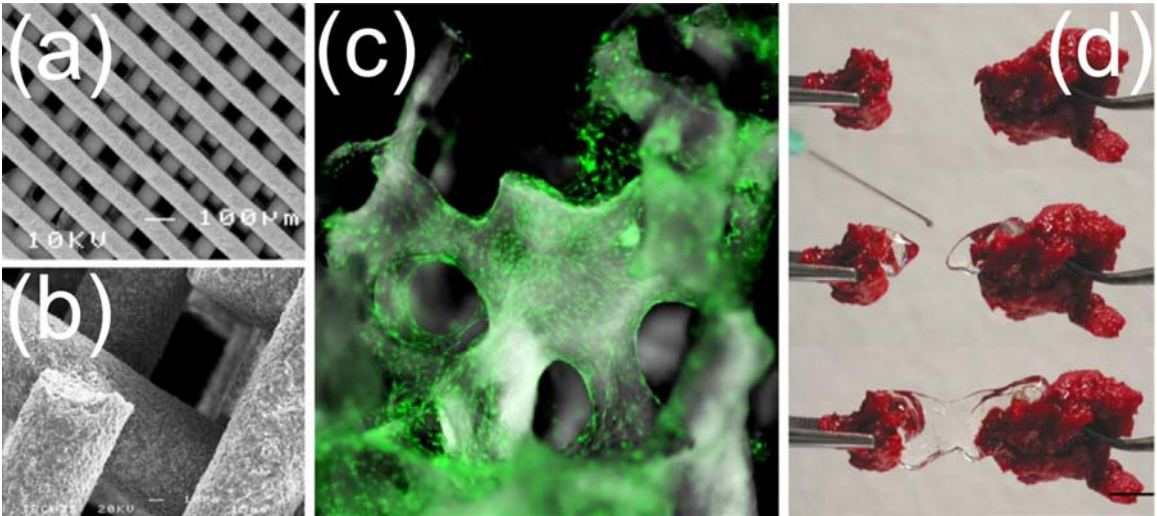


FIGURE 16.14
Examples of scaffolds for bone regeneration.

respective similarity to the organic and inorganic components of natural bone tissue. While biocompatibility is more of a concern with synthetic polymers, biodegradable polymers, such as poly(L-lactic acid), poly(glycolic acid), poly(D,L-lactic-co-glycolic acid), and poly(ε-caprolactone), are already widely used for certain biological applications. Additionally, various other polymers such as polyorthoester, polyanhydrides, and poly(propylene fumarate) are being investigated for tissue engineering and have shown favorable biocompatibility in animal models.⁵⁹

Biodegradability—The degradation of a tissue engineering scaffold is to be carefully controlled so that scaffold resorption and bone formation are tightly coupled. While, on the one hand, persistence of a graft material can result in poor clinical outcomes, especially when bone integration is poor, premature resorption of a scaffold also compromises bone repair by the destabilization of early bone formation and the loss of osteoconductive surfaces for new bone apposition.⁶⁰ Controlled resorption rate is thus a key parameter in bone tissue engineering.

Mechanical properties—A scaffold for bone tissue engineering should approximate the mechanical function of the callus in natural bone repair, and provide a dynamically durable-degradable 3D structure, which, over time, can be replaced by cell-derived tissue function.⁶¹ While weight-bearing functionality may be an ideal in certain contexts, it is not a prerequisite specification of a bone tissue engineering scaffold (especially if resorbability or mechanical signal transmission is thereby compromised) as support can be achieved via alternative orthopedic techniques.⁶²

Porosity—Scaffold porosity (percentage void space in a solid), pore size, and pore interconnectivity impact on the surface area available for cell growth and accessibility for host tissue, including vasculature, to penetrate into the central regions of the scaffold.⁶² Increase of porosity and pore size has been found to positively influence bone formation, pore interconnections <100 μm have been found to restrict vascular penetration, and supplementation of a porous structure with macroscopic channel-enhanced tissue penetration and bone formation.⁶³

Scaffold as ECM—In addition to providing appropriate mechanical support at the defect site, the tissue engineering scaffold serves to reestablish a regenerative microenvironment at the site of damage by providing the dynamic extracellular signaling—chemical, biological, and mechanical—that directs progenitor cell recruitment, growth, and differentiation. Bone ECM itself provides a ready example of the dynamic role the ECM plays in biological signaling. Early work in the 1960s identified BMPs as the active factors responsible for the ability of demineralized bone matrix (DBM) to induce ectopic bone formation upon transplantation.⁶⁴ However, while recombinant BMPs (rhBMPs) have now been clinically approved for the induction fracture repair, BMP-based strategies require milligram doses of the protein for reliable fracture repair in contrast to the micrograms of BMP per kilogram of bone graft.

In addition to controlling the diffusion of chemical and biological signals, the ECM is also associated with directing cell growth and differentiation via direct interaction with cell surface receptors. Thus, for example, Type I collagen, the major organic component of bone ECM, is chemotactic to fibroblasts possessing high-affinity cell-binding domains, and type I collagen-specific binding is known to mediate the osteogenic response of human BMSCs. Synthetic polymers may be modified by the attachment of certain peptides derived from ECM proteins. One such peptide, Arg–Gly–Asp (RGD), is derived

from the ECM proteins fibronectin and laminin, and is widely studied for promoting biomolecular recognition, cell attachment, and cell function.

16.31 The function of the vasculature in skeletal regeneration

The bone vasculature is a dynamic, complex, ordered system influencing growth, homeostasis, and repair of the skeleton. It has the critical and complex function of continuously orchestrating blood flow to the skeletal tissue, responding rapidly to changes in the tissues vascular environment while intrinsically balancing the spatial–temporal vascular–skeletal homeostasis. Blood vessel formation and osteogenesis are inherently linked in building the skeletal system thorough two processes: intramembranous and endochondral ossification.⁶⁵ The bone vasculature provides an essential transport conduit for oxygen, nutritional factors, host stem cells, hormones, immunity, and the removal of waste products. It provides vascular niches for hematopoiesis, as well as the necessary intrinsic cointegration of vascular and skeletal cells to release biofactors that regulate skeletal growth and regeneration.⁶⁶ It is well characterized that endothelial cells that line all blood vessels (endothelium layer) play an integral role in many physiological processes including the differentiation of osteogenic progenitors.⁶⁷

Detailed analysis of endothelial populations in bone has revealed a number of subtypes with unique functional roles. The vascular niche of bone has been characterized to contain specific endothelium (Type-L or Type- H) expressed in defined regions of bone.⁶⁸ In addition, a third type (Type-E) associated with the developmental stages of murine bone has also been identified.⁶⁹ These specific endothelium of bone have specialized functional roles identified in their signaling cascades in the vascular niche interacting and modulating with hematopoietic and MSCs, osteoprogenitors, pericytes, macrophages, and osteoclasts.⁷⁰

A number of key osteogenic and angiogenic growth factors are required for fetal and adult bone development, and clinical repair of bone fractures.⁶⁵ One of these factors is the potent angiogenic inductive growth factor, VEGF. VEGF plays a key role in intramembranous and endochondral ossification. Its secretion is integral to endochondral skeletal development where it induces angiogenesis, chondrocyte apoptosis, stem cell differentiation, and the ossification of the growth plate.^{65,71} This cascade of effects is essential for the successful repair of bone fractures. VEGF, a prosurvival factor for endothelial cells along with hypoxia, can elevate the levels of BMP-2 expression in vascular endothelial cells. Furthermore, bone marrow–derived MSCs that overexpress HIF-1 α have the ability to heal critical-sized bone defects, with HIF-1/VEGF pathway overexpression increasing bone regeneration rates.⁷² Conversely, BMPs, a potent inducer of bone formation, can enhance the production of VEGF-A in osteoblasts with increased angiogenesis. Indeed, BMP-2 combined with FGF-2 and VEGF can enhance endothelial angiogenesis, and the combination of BMP-2/VEGF has the ability to influence stem cell homing and differentiation leading to enhanced bone regenerative outcomes.⁷³ Critically, in the bone repair cascade of fracture models, angiogenesis is induced prior to the onset of programmed osteogenesis; however, interventions to reduce angiogenesis and levels of VEGF can lead to significant healing impairment of the bone fracture.

The endothelial cell and osteoprogenitor cell can influence each other's functional processes.⁷⁴ With the identification of specific subsets of microvascular endothelium in bone, understanding their cross-talk

and the osteogenic–angiogenic coupling with MSCs and osteoprogenitors is fundamental to maintaining the integrity of the skeleton allowing the identification of better therapies for bone tissue regeneration. Combinations of vascular and osteogenic cell implantations have reported successful bone fracture repair in a critical sized defect using a tissue engineered alginate microsphere delivery system.⁷⁵ With the advancement of biofabrication and bioprinting technologies, new functional materials for bone repair via the combinatory angiogenic/osteogenic are successfully being developed. Prevascularized hybrid scaffolds of adipose stem cells (ASCs) and HUVECS have successfully been demonstrated to repair critical sized bone defects.⁷⁶ Furthermore, complex bio composites with the ability to release dual growth factors (i.e., VEGF and BMP-2) in combination with seeded MSCs at the same release rate or at staggered release intervals at concentration levels compatible to that expected in vivo bone fracture repair have been demonstrated to enhance fracture repair with increased vascularization.⁷⁷

Recent advances in vascular analysis, such as the identification of bone endothelium subsets, in vivo vascular imaging probes, vascular perfusion contrast agents, microcomputed tomography, and the use of the chorioallantoic membrane (CAM) assay model (Fig. 16.15), are now providing us with

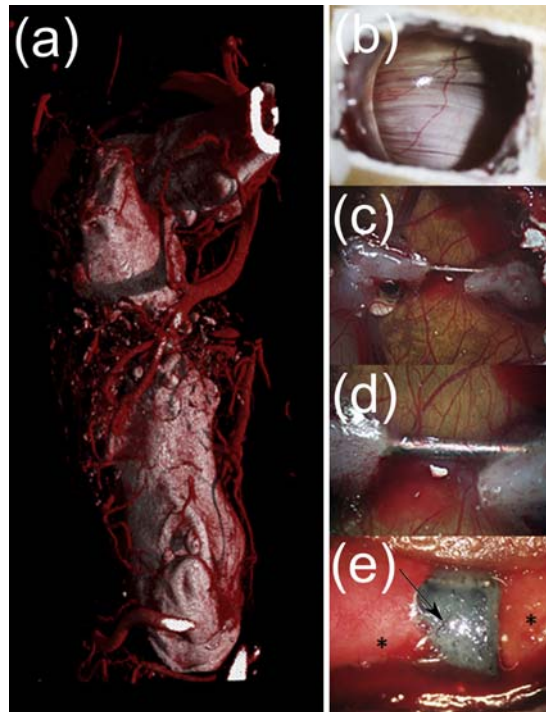


FIGURE 16.15

The three-dimensional image (μ CT) of perfused contrast vascular dye (red) depicting the blood vessels in a critical sized mouse femur defect (a). The window of a D18 chick egg depicting the vasculature of the CAM (b). D18 chick femur defect stabilized with a 26 G needle after 8 days of implantation in the CAM: (c) low magnification, (d) high magnification. D18 chick femur defect with an implanted bioscaffold in the defect region (e) (arrow = blood vessel; * = bone) after 8 days of implantation in the CAM.

valuable information on the direct and indirect roles of the vasculature in bone fracture repair leading to alternative strategies to utilize angiogenic–osteogenic coupling. One such delivery system is the incorporation of gene-modified cells into scaffolds resulting in the spatio-temporal expression of factors of interest. BMP2-VEGF transfected into ASCs in a 9:1 ratio using an electroporation approach were able to regenerate bone when implanted into critical sized bone defects both in the limb bone and the calvaria.⁷⁸

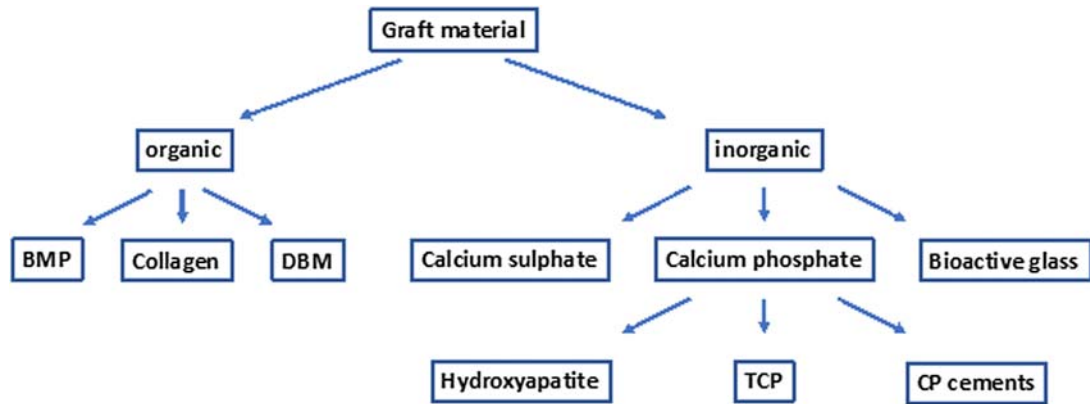
Finally, direct mechanical stimulation can have an impact on vascular development and subsequent bone repair. Thus, the ability to scale up bioengineered cell-scaffold constructions for clinical application, with optimal biomechanics, vascularization, and osteoinductive capacities will be critical in the deployment of vascular bone graft alternatives in the future.

16.32 Animal models in bone tissue engineering

Animal models form an essential link in the development of bone tissue engineering solutions. Animal models can be categorized into three areas of use⁷⁹: (1) High throughput “fundamentals” research, which typically uses small mammal laboratory species: mouse, hamster, and rat. These species are suitable for large study sizes and are the staple elements for early stage in vivo models; these offer ease of handling, reduced costs, and ready availability through commercial channels. Adversely, short study duration and considerable dissimilarities biomechanically and physiologically limit the clinical relevance of outcomes. (2) Comprehensive, longer-term bioactivity and feasibility studies favor classically medium-sized species such as rabbit, dog, and goat. Studies inform further translation principally through a scale-up in body mass, increase in defect volumes, and marginal scope for incorporating application specific scenarios. However, quadrupedal gait, the lack of analogous secondary bone remodeling, and poor experimental tolerance are issues to consider. Although not without merit, midsized in vivo models cannot inform to the same degree of accuracy as large mammal studies and, as such, are typically considered where facility limitations dictate or as suitable preclinical trial model. (3) Late-translational stage clinical modeling, designed to trial a solution in a scenario as close as is possible to its clinical application. This stage is almost exclusively the domain of ovine models with a limited number of studies using bovine and porcine species. Models in this category are most accurate for assessing clinical efficacy due to analogous species weight, biomechanics, and bone structure with humans. Increased body size and mass allow design protocols matched to human procedural protocol and negative disparities resulting from a quadrupedal gait, although experimentation is typically costly with significant infrastructural requirements often restricting study number.⁸⁰

16.33 Clinical experience in bone tissue engineering

Trauma and orthopedic surgeons have utilized bone tissue engineering products to improve patient outcomes in conditions ranging from cervical spinal arthrodesis (spinal fusion) to fixation of calcaneal fractures. Traditional bone grafting options derived from natural products include autograft in which bone is taken from the patient themselves, and allograft which is cadaveric derived bone. With a few rare exceptions such as a vascularized fibular graft, autograft and allograft are avascular.

**FIGURE 16.16**

Examples of different organic and inorganic graft materials.

Key bone graft properties include osteogenicity, osteoinductivity, osteoconductivity, and mechanical strength.⁸¹

Osteogenicity is bone synthesis from cells within the graft, while **osteoinductivity** describes the ability of graft to recruit and induce cells to become osteoblasts. **Osteoconductivity** quantifies the properties of the graft as a scaffold for cellular migration, in which porosity and cell adherence are critical properties.

An autograft remains the clinical gold standard and is unique in regard to osteogenic properties. Autograft material is typically resected from the iliac crest, a secondary operative procedure. Quantity of autograft harvestable is strictly limited, usually results in pain, and occasionally infection. While allograft avoids these complications, allograft is not osteogenic, may mediate inflammation at the target site, and may potentially transmit disease.

A variety of surgical requirements in conjunction with the limitations of autograft and allograft have stimulated the development of a range of regenerative bone graft products.⁸¹

Bone graft products exist as putty, paste, granules, or cement and are derived from organic and inorganic materials. The material properties from which synthetic bone graft is derived are discussed following which surgical requirements and clinical use are evaluated (Fig. 16.16).

BMP is produced using recombinant gene technology and is osteoinductive, while the type 1 collagen is typically bovine derived and osteoconductive. Demineralized bone matrix (DBM) is an allograft, in which acid is utilized to remove the mineral component, and the remaining protein mix subject to some form of sterilization procedure such as gamma irradiation or ethylene oxide. Contents of DBM include type 1 collagen, BMP, and TGFβ. While DBM is both osteoinductive and osteoconductive, it provides no mechanical strength and is extremely variable in clinical effect, presumably due to donor variation.

Calcium sulfate provides a biocompatible osteoconductive scaffold; however, rapid resorption results in early loss of integrity and strength. Conversely, pure hydroxyapatite (HA), $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, with

the CP stoichiometric ratio of 1.67 undergoes extremely slow resorption and is usually maintained at the target site for years. Properties of HA including the osteo inductivity, conductivity, and resorption are partly determined by minor substituents such as magnesium, sodium, and carbon. Porosity quantifies pore size, volume, and degree of interconnectivity, with the ideal porosity for graft material matching that of cancellous bone. Interconnecting macroporosity is required for vascularization and bone ingrowth thereby facilitating graft resorption. Manufacturing processes offer the opportunity to control HA porosity allowing biomechanical optimization for clinical use.

Tricalcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ (TCP) can exist as two crystalline forms, with β -TCP the form used for synthetic bone. β -TCP has porosity similar to cancellous bone and is typically resorbed within months.

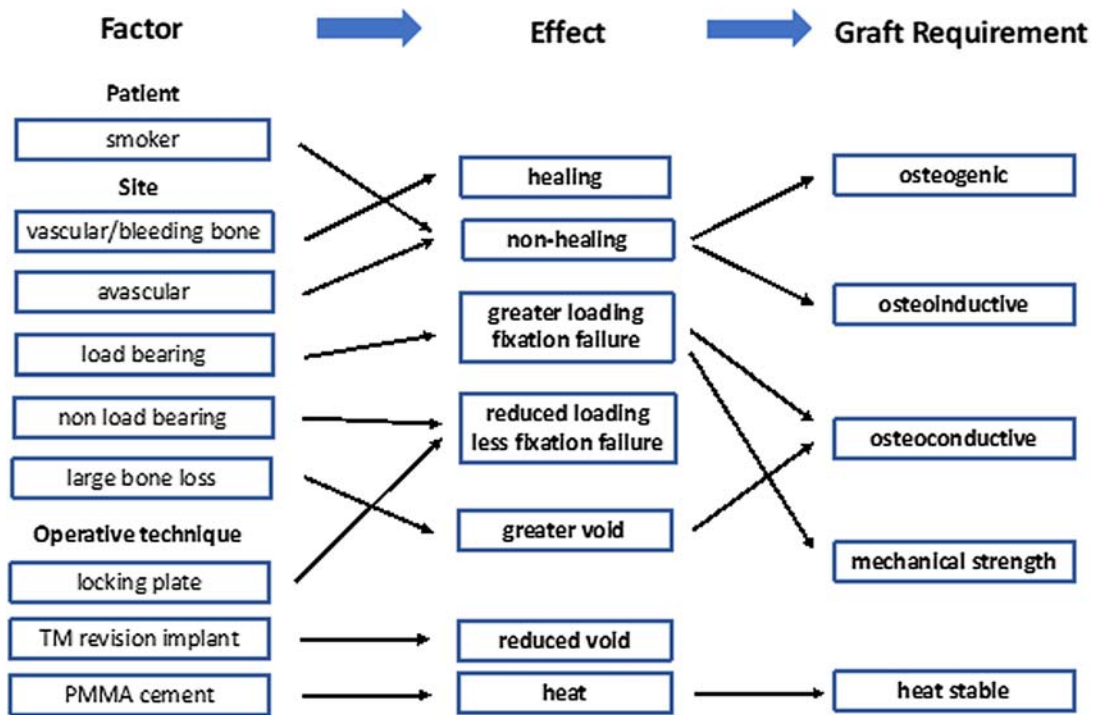
CP cement is a combination of β -TCP, calcium carbonate, and monocalcium phosphate. First developed by Brown and Chow (US Patent No. 4,518,430) several formulations are now available. CP cement hardens in an iso-thermic reaction to produce an HA like material which is essentially nonresorbable and offers little tensile strength.

Bioactive glass is both osteoinductive and osteoconductive, composed of metallic oxide materials of which the main component is silicon dioxide. The glass is termed bioactive due to the ability of the material to stimulate HA formation on the surface when in contact with bone without generation of an interposing fibrous layer.

Dozens of synthetic bone graft products are available from a variety of companies.⁸¹ In many cases, products combine two or more of the materials described to enhance the biomaterial properties for clinical use. Materials composed of two forms of CP are termed biphasic; for example, OsSatua is composed of 80% HA and 20% β -TCP, the latter material undergoes more rapid resorption, allowing vascular ingrowth while maintaining mechanical strength due to the persistence of HA.

From the surgical perspective, critical properties of the synthetic graft material are determined by the interplay of patient factors including smoking and osteoporosis, local factors such as soft tissue damage, vascularity, void size and mechanical loading, and surgical technique including stabilization with locking plate or exothermic polymethylmethacrylate cement (Fig. 16.17).

The operative site in scaphoid fractures is poorly vascularized, predisposing to nonunion; however, mechanical loading during healing is easily avoided. Thus, merely biological stimulation may be required in treatment of nonunion which can be accomplished with the use of BMP-2⁸² without recourse to graft material. Conversely, nonunion is rarely a problem in comparably well-vascularized tibial plateau fractures. A key challenge in management of tibial plateau fractures is avoiding collapse of the articular surface in this load bearing lower limb joint. Thus, the primary graft requirement is provision of mechanical stability thereby permitting weight bearing without articular collapse.⁸³ Biphasic HA calcium sulfate cement⁸³ and bioactive glass granules⁸⁴ have shown equivalent clinical and radiographic outcomes to autograft when used with locking plates in treatment of tibial plateau fractures. It may be inferred that osteogenecity afforded by autograft compared to synthetic osteoconductive material offers no additional benefit to healing as the fracture is surrounded by bleeding cancellous bone at this anatomical site.

**FIGURE 16.17**

Matrix that indicates how patient and surgical requirements determine the graft's biological response.

In addition to factors related to the operative site, selection of operative technique also determines in part the graft requirements. For example, the risk of fixation failure can be mitigated through the use of locking plate systems, in which the screw head locks into threads within the plate producing a rigid poly axial construct. With additional stability provided by the fixation system, the construct is less dependent on provision mechanical stability from the bone graft material. Kim et al.⁸⁵ found no advantage in maintaining distal radius fracture reduction when using CP cement in combination with a volar locking plate compared to locking plate fixation alone. In addition, leakage of bone cement from the target site, a particular risk in comminuted (multifragmented) distal radius fractures has been reported and can necessitate additional surgery.

In some situations, improvements in implant technology have totally removed the need for graft material in reconstructive surgery. For example, bone defects encountered during revision hip arthroplasty which historically required allograft are now often reconstructed with trabecular metal implants.⁸⁶

Spinal arthrodesis describes the inherently challenging process of getting two or more vertebrae to fuse together and often requires the use of iliac crest–derived autograft (ICBG). A vast array of bioactive glass and CP-based graft products have been deployed alone and in combination with adjuncts such as locally derived autograft or bone marrow aspirate in lieu of ICBG. While scores of trials have

compared effectiveness of synthetic bone graft products with ICBG, variations in surgery, patients, grafts, and adjuncts make comparisons challenging. In general terms, several studies have demonstrated that bioactive glass formulations⁸⁷ and CP-based products,⁸⁸ when combined with locally derived autograft as an adjunct, result in fusion rates equivalent to those seen with ICBG. This is of clinical significance as it avoids a secondary procedure of ICBG and associated risks.

While these studies suggested use of an adjunct to the graft was essential to promote osteogenesis, a recent randomized controlled trial demonstrated efficacy of a standalone CP graft was not inferior to autograft in mediating posterolateral lumbar spinal fusion.⁸⁹

For some time, fixation devices have been designed to accommodate graft material, with spinal cage systems as a prominent example. It is likely that the future will yield increasing synergy of fixation devices and implants with bioengineering strategies. Employment of a bioabsorbable screw designed for cruciate ligament reconstruction in management of tibial plateau fractures⁹⁰ exemplifies total integration of a fixation system with synthetic bone graft material.

In summary, despite recent advances in bone engineering strategies, the rate-limiting step remains the vascularization of graft material postimplantation. It is this essential component upon which osteointegration and fracture healing depend.

16.34 Future perspectives for bone regeneration

The relative accessibility of an autologous osteoprogenitor population has led to progress and considerable interest in the application of SSC therapy in the clinic hampered predominantly by our limited understanding of SSC fate, immunophenotype, and selection criteria in the absence, to date of specific selective markers. While pluripotent cells, with their ability to differentiate into any cell of the three germ layers (endoderm, mesoderm, ectoderm), are an attractive approach in regenerative medicine, embryonic stem cells hold limitations in their use given their allogeneic nature and ethical issues in their application. More recently, induced pluripotent stem cells (iPSCs) offer an autologous approach, derived from the reprogramming adult somatic cells and yet, concerns around inherent tumorigenicity, potential genetic, and epigenetic variants present a significant clinical hurdle to be resolved. From a clinical translation and commercialization perspective, allogeneic therapies are attractive if the therapeutic cell fractions can be readily sourced and used “off-the-shelf,” with the attraction of a single universal donor approach. However, the risks of immune rejection and iatrogenic infection mean that autologous therapies are often preferred for translation even if such cells may retain some of the defects leading to the original disease in the individual.

Within the biomaterials landscape, materials that can provide cell cues whether through topography, surface roughness, mechanosensitive cues, spatial cargo delivery, or programmed scaffold degradation facilitating tissue growth and cargo release will undoubtedly come to the fore. Material properties and cell-induced changes/interactions align closely with the need to marry increased understanding of the importance of harnessing the immune system and the interactions between proinflammatory (M1) and antiinflammatory (M2) states to modulate and guide the reparative process. For routine and, critically, robust bone formation approaches, the future challenge for regenerative medicine will center on the ability to scale up bioengineered vascularized grafts/materials for clinical applications. However, a

constant remains, the successful repair of large osseous defects or the integration of any other large regenerative tissue constructs will be only as good as the development concomitantly of a functioning blood supply.

The future is bright for regenerative medicine with the emergence of new state-of-the-art molecular and biological tools (iPSCs, CRISPR/Cas9, optogenetics, omics technologies/imaging platforms), in vitro models, additive manufacturing (AM), and artificial intelligence/machine learning to inform the mechanisms involved in tissue regeneration.^{91,92} Future approaches will undoubtedly be centered on the development of simple, safe, and efficacious protocols of cell expansion that will meet stringent regulatory body criteria for clinical applications. This approach combined with bioinformatics will generate a phenotypic fingerprint of the skeletal stem progenitor cell at a single cell resolution. This will be followed by derivation of skeletal cell populations from pluripotent stem cell sources. Ultimately, approaches will include the development and integration of immunoprivileged constructs containing an appropriate scaffold/growth factor(s) composition for autologous and potentially, allogeneic skeletal populations facilitating immune evasion, and delivering clinical application for an increasing aging population.

Summary

- Adult articular chondrocytes exhibit a level of phenotypic plasticity that is comparable with that of MSCs.
- The number of chondrocytes needed to start chondrogenesis in a scaffold is $>20 \times 10^6$ cells or at least 1×10^6 cells/cm² of cartilage defect area.
- There is a need to study the use of freshly isolated chondrocytes for direct implantation.
- Pooling of zonal chondrocytes from different regions is an acceptable source for chondrocytes to be used for cartilage tissue engineering.
- There is, as yet, no ideal scaffold for cartilage engineering. In small defects in vitro, developed mature osteochondral constructs may be used in the future instead of autologous osteochondral plugs with or without cells. In larger defects, hyaluronan-based scaffolds in combination with chondrocytes are an option that could be further evaluated.
- Small- to medium-sized traumatic defects need to be treated differently compared to large osteoarthritic defects, where the cartilage engineering process has to be directed not only to fill a defect but also to withstand the negative influence of a diseased joint. The tissue engineering approach should address several phenomena including
 - blocking the production of proinflammatory factors and suppressing the progression of the degenerative process affecting both cartilage and the bone compartment.
- Bone is unique and has a capacity to renew and remodel throughout life. Bone serves a number of key functions that include structural support, protection of internal organs, locomotion, maintenance of mineral balance, and as a reservoir for growth factors and for hematopoiesis.
- Bone marrow contains the SSC that provides the inherent regenerative capacity of bone. These skeletal stem and progenitor cells are typically identified by using a panel of antibodies by applying either positive or negative immunoselection; however, to date, a single unique cell surface marker for the SSC remains elusive. The SSC has been characterized as residing within the perivascular spaces around the vascular sinusoids in the bone marrow and, critically, a single SSC is capable of generating a complete heterotopic bone or bone marrow organ-ossicle including establishment of host hematopoiesis.
- The precise identity of the regulatory effects of SSCs on immune and inflammatory cells remains to be validated conclusively in a defined in vivo system.

Continued

Summary—continued

- Bone formation involves the exquisite spatiotemporal regulation of recruitment and directed differentiation of bone cell populations under the control of a range of growth factors. A number of osteoinductive growth factors have been identified, including BMP-2 and BMP-7.
- 3D matrices, or scaffolds, form a vital component of bone tissue engineering. Ideally, the scaffold design and properties should align with the mechanical properties of the bone it will replace and should be resorbable. Cancellous bone autograft provides an osteoconductive, osteoinductive material for orthopedic application; however, limitations in supply and requisite surgery (and complications therein) have led to extensive research to derive alternative materials for clinical applications.
- Critical in any bone tissue regeneration development is a functional vascular system to provide transport of oxygen, nutritional factors, host stem cells, hormones, immunity, and the removal of waste products as well as vascular cells and skeletal cells to aid skeletal growth and regeneration. Development of cell-loaded, vascularized constructs for application in large segmental bone defects or large bone reconstruction remains an unmet challenge.
- The future challenge for regenerative medicine will center on the ability to scale up bioengineered grafts for clinical applications, including an appropriate cell source and vascular supply.
- New approaches in skeletal regenerative medicine will harness state-of-the-art molecular and biological tools (iPSCs, CRISPR/Cas9, optogenetics, omics technologies/imaging platforms), in vitro models, additive manufacturing, and artificial intelligence/machine learning to inform the mechanisms at play in tissue regeneration and drive clinical application.

Classical experiment

Urist—BMPs

In the 1960s, Marshall R. Urist⁹³ investigated decalcified bone that was implanted at various locations, both heterotopic and homotopic, in several types of animals and in humans. These acellular, decalcified bones (i.e., only the organic part of bone tissue) yielded new bone in amounts proportional to the volume of the implant, with over 90% positive experimental results. Evaluation using light microscopy showed that initially (3 weeks postimplantation) these implants were enveloped in highly vascular, inflammatory, and fibrous connective tissue. Subsequently, invading cells of the host started the process of resorption of the collagenous matrix, after which new bone deposits became apparent after 4–6 weeks. These early deposits were always found in the interior of well-vascularized excavation chambers. Also, cartilage formation was observed in the closed ends of old, vascular channels, which was gradually replaced by bone tissue between eight

and 16 weeks via the process of endochondral ossification. At this point, Urist called this process “osteogenesis by autoinduction.” In later experiments, Urist et al.,⁹⁴ introduced the term BMP for a proteinaceous compound that was obtained by chromatographic separation of a collagenase digest of bone matrix gelatin. This compound was loaded into diffusion chambers, which were implanted in muscle pouches in adult rats, and demonstrated to induce osteogenesis in the direct vicinity of the diffusion chamber. The purification and characterization of BMPs became established in the late 1980s, as did their amino acid sequence and DNA clones.⁹⁵ Currently, many different types of BMPs have been identified, which differ in function.⁹⁶ Although the yield of fresh BMPs out of bone is extremely low (10 kg of bovine bone powder yields 10 µg of BMP), recombinant DNA technologies have allowed now the production of large quantities of the so-called rhBMP for commercial exploitation and clinical use. An

Classical experiment—continued

example of the in vivo potential of rhBMP-2 on porous CaP implants in rabbits is shown below. Several clinical reports on nonunions in tibia, femur, and the spine have also shown beneficial results for the use of rhBMPs. Spine fusion models, such as an anterior (cage) or posterolateral approach (both with or without instrumentation), have shown additional positive results for the use of both rhBMP-2 and OP-1 (rhBMP-7) as an alternative to the use of autograft bone. Several studies have measured both clinical signs of fusion (such as the Oswestry pain and disability scoring system) and radiographic evidence for successful fusion. Unfortunately, large quantities of BMPs of up to 20 mg per side (posterolateral fusion model)

are needed with subsequent considerable financial consequences.

Urist MR. Bone: formation by autoinduction. *Science*. 1965; 150:893–899.

Urist M, Nogami H, Mikulski A. A bone morphogenetic polypeptide. In: *Calcified tissues* 1975. Springer; 1976.

Wozney JM. The bone morphogenetic protein family and osteogenesis. *Mol Reprod Dev*. 1992;32:160–167.

Rengachary SS. Bone morphogenetic proteins: basic concepts. *Neurosurg Focus*. 2002;13:1–6.

State-of-the-art experiment

Nanoclaybased 3D printed scaffolds promote vascular ingrowth and bone formation

The ideal bone graft material should match the microarchitecture and biomechanical properties of bone. Layer by layer directed synthesis observed in 3D printing (3DP) permits precise control of fiber alignment and porosity, offering the potential to control the microenvironment for bone regeneration.⁹⁷ Stimulation of bone has been achieved in clinical practice with BMP although adoption of this technology has been limited due to inefficient delivery mechanisms. The nanoclay LAPONITE ($\text{Na}_h(\text{Mg}_{3-h}\text{Li}_h)\text{Si}_4\text{O}_{10}(\text{OH})_2 \cdot n\text{H}_2\text{O}$), a synthetic smectite (herein, Laponite), has shown promise as a delivery vehicle for BMP⁹⁸ and VEGF.⁵⁶ Laponite is particularly suitable for bioprinting as the thixotropic (sol-gel) nature permits transit through a nozzle and later restitution of shape. Laponite also has an inherent⁹⁹ and indirect⁵⁶ ability to stimulate

osteoblastic differentiation of human bone marrow stromal cells (HBMSCs).

The use of nanoclay in 3DP represents an exciting new development in the field of regenerative medicine offering the opportunity to combine the inherent advantages of 3DP with the unique properties of Laponite. A Laponite-alginate-methylcellulose bioink was produced at 3% w/v (3-3-3 scaffold) and printed using a bioprinter to produce $10 \times 10 \text{ mm}^2$ scaffold. HBMSCs encapsulated in the bioink prior to printing were observed to remain viable and functional. Vascularization of the 3DP scaffold was enhanced with VEGF and human umbilical vein endothelial cells (HUVECS) in a chick CAM assay (Fig. 16.18a). Bone formation within the 3DP scaffold was shown to be greater than alginate control and enhanced with BMP in a subcutaneous murine model Fig. 16.18b. This study demonstrated the ability of 3DP Laponite-based gel to convey functional

Continued

State-of-the-art experiment—continued

HBMSCs and localize both VEGF and BMP. Further development will enable this technique to provide a microarchitectural environment matched to bone and provide

spatially controlled delivery of cells, VEGF and BMP optimized for bone regeneration tailor-matched to a defect site.

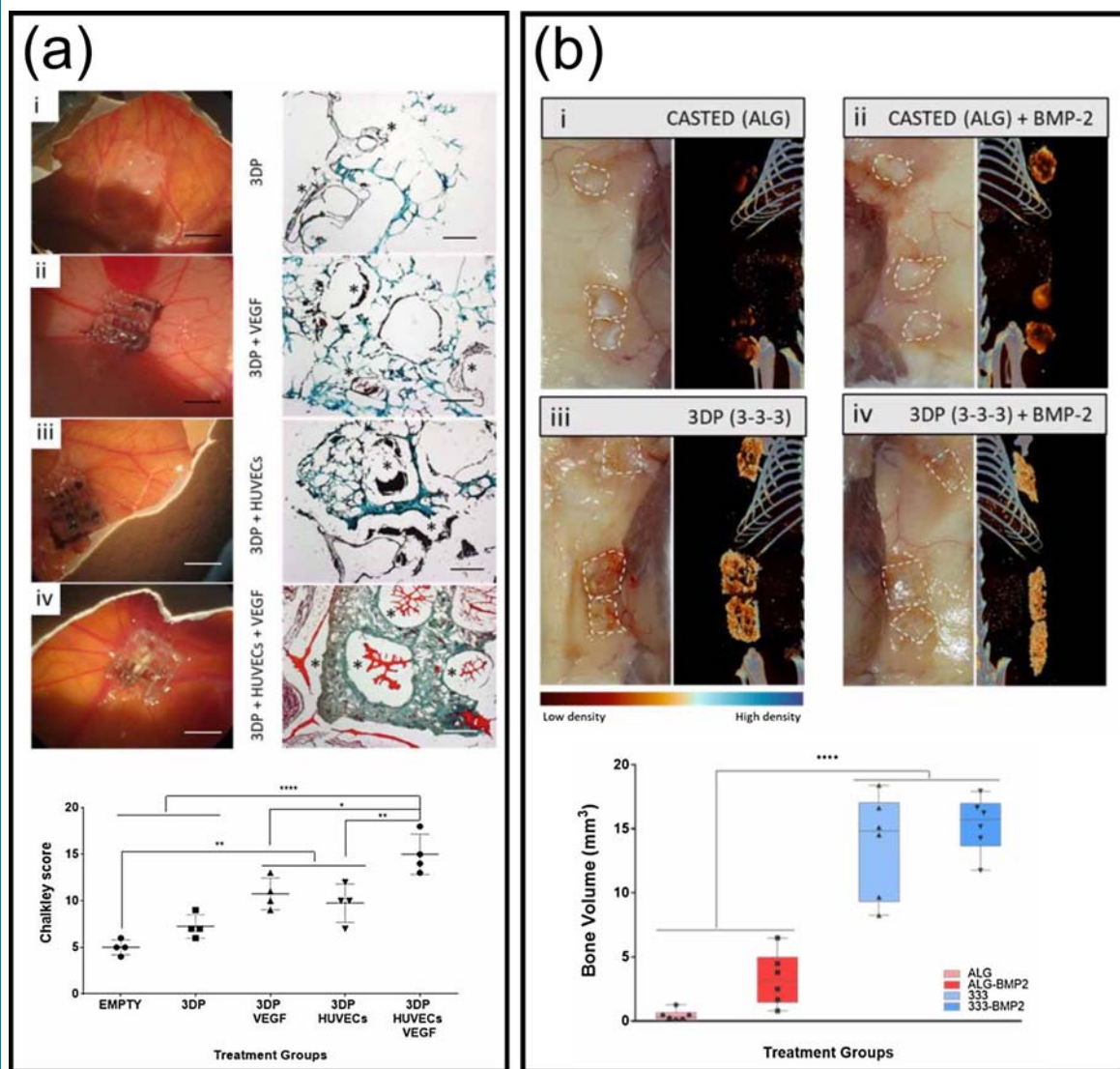


FIGURE 16.18

(a) Vascularization in a chick chorioallantoic membrane assay observed in 3DP (3,3,3) scaffold alone and with: VEGF, HUVEC, and both VEGF and HUVEC. (b) Bone formation on 3DP(3,3,3) scaffold alone and in combination with BMP in a subcutaneous murine model with alginate control. From Cidonio et al. *Nanoclay-based 3D printed scaffolds promote vascular ingrowth ex vivo and generate bone mineral tissue in vitro and in vivo*. Biofabrication. 2020 May 12;12(3):035010. <https://doi.org/10.1088/1758-5090/ab8753>.

16.35 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
1. What type of collagen is predominantly found in hyaline cartilage?
 2. What is the most abundant proteoglycan found in cartilage?
 3. Which cells are responsible for cartilage matrix synthesis?
 4. What happens to chondrocytes when they are cultured in 2D monolayer cultures?
 5. From which anatomical locations can chondrocytes be isolated?
 6. Which technique is recommended by NICE for the repair of chondral defects over 2 cm²?
 7. What is the minimum chondrocyte seeding density required to start chondrogenesis in a scaffold?
 8. Name three functions of bone.
 9. What is located between the epiphysis and the diaphysis? What cell serves as a mechanoreceptor in bone?
 10. What cell degrades bone matrix?
 11. What is the main type of collagen in bone?
 12. Name an osteoinductive growth factor.
 13. What predominantly forms the mineral phase of bone?
 14. Name types of fracture healing.
 15. Name two types of bone formation.
 16. Is woven bone anisotropic or isotropic?
 17. Name types of bone graft derived from humans.
 18. Define a critical size defect.
 19. Name types of material used in synthetic bone graft products.
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:
1. Describe the structure of articular cartilage and explain how it functions as a shock absorber in the joints.
 2. Describe the autologous chondrocyte implantation technique, list the NICE guidelines for its use and the limitations of this technique.
 3. Describe the structure of bone.
 4. Describe the process of fracture repair.
 5. Describe the isolation and characterization of skeletal stem cells.
 6. Discuss the use of scaffolds in bone regeneration.
 7. Discuss the function of the vasculature in skeletal regeneration.
 8. Characterize the properties of bone graft material.
 9. Describe the materials from which synthetic bone graft products are derived.
 10. Compare and contrast the likely bone graft requirements in fixation of a scaphoid nonunion and a tibial plateau fracture.

Challenge-based learning

Design of a smart scaffold with a bioelectronic sensor to sense BMP-2 release and bone formation in bone grafts

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Small bone defects can be treated with bone graft materials and recombinant growth factors but the golden standard for the treatment of large bone defects is the autograft, in which bone is moved from one part to another within the body. Many engineers attempt, and succeed, to improve the osteogenic potential of graft materials but assays to predict in vivo bone formation using in vitro assay are unreliable. Animal experiments and imaging are the only way to conclusively demonstrate the efficacy of a novel graft. In these models, bone formation is an endpoint measurement, in which the tissue is surgically removed from the euthanized animal after weeks to months of implantation to evaluate bone quality. This totally ignores the dynamics of tissue formation and leaves the researcher with little information about the in situ bioactivity of the grafts. The long-term vision is to design experimental grafts which are able to monitor the bone formation process in living animals.

Motivation and stakeholders

The field of bioelectronics has many opportunities, with ever smaller size of the electronics and incorporation of molecular analysis. There is an opportunity to design biosensors that rely on electrical signals generated once the bone is loaded and unloaded to unveil bone formation and quality. Newly designed grafts to monitor in situ bone formation should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as orthopedic surgeons, biomaterial engineers, and microelectronic experts.

Problem definition

Bone morphogenetic protein-2 (BMP-2) is a growth factor involved in bone formation, and it has been applied for spinal fusion therapy for years now. However, the amount of BMP-2 needed to achieve fusion far exceeds physiological levels. Still, the use of BMP-2 and other growth factors may eventually also substitute autologous grafting, but for this, its in vivo bioactivity should be closely monitored.

Challenge

To design a bone graft using electrical signals generated when the bone is loaded/unloaded which is able to monitor BMP-2 activity and bone formation in situ.

Learning framework

Reading the Bone Tissue Engineering chapters and related literature will help you to understand the following:

1. Critical size bone defects.
2. Graft materials used to treat large bone defects.
3. The use of BMP-2 as a bone-inducing growth factor.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

4. Bioelectronics and which biomaterials are conducive to biosensors.
5. Manufacturing of bioelectronic sensors.
6. Current techniques used to study the release of BMP-2 in tissue microenvironment.
7. Biomarkers in the tissue microenvironment to monitor BMP-2 activity

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

16.36 Glossary

Allogeneic means cells, tissues, or organs derived from antigenically dissimilar individuals from the same species.

Articular cartilage is a specialized connective tissue located at the end of long bones.

Autologous means cells, tissues, or organs derived from the same individual.

Autologous chondrocyte implantation is a tissue engineering strategy in which articular cartilage cells are collected, expanded, and reimplanted into a cartilage defect.

Cancellous bone also called trabecular bone, is the meshwork of trabecular tissue of adult bone found at the ends of the long bones or at the core of vertebrae in the spine.

Chondrocytes are the only cells found in healthy cartilage with the function to produce and maintain the cartilaginous matrix.

Chondroinductive means the property to induce cartilage growth.

Chondron is the combination of a chondrocyte and its associated pericellular microenvironment.

Cortical bone refers to the outer hard layer of the bone.

Critical size defects are bone defects typically larger than 2.5 cm that will not heal within a patient's lifetime.

Dedifferentiation is a process by which cells become less specialized and return to an earlier cell state within the same lineage.

Endochondral ossification is a process of bone formation in which bone replaces a cartilage matrix.

Induced pluripotent stem (IPS) cell is a type of pluripotent stem cell generated directly from a somatic cell by expressing the transcription factors Oct 4, Sox2, Klf4, and c-Myc.

Intramembranous ossification is a process in which bone is laid down by direct apposition within stromal connective tissue.

Microfracturing is a bone marrow stimulation technique achieved by subchondral bone perforation to recruit autologous mesenchymal stem cells to a cartilage defect.

Osteoarthritis is a type of degenerative joint disease that results in the breakdown of joint cartilage and the underlying bone.

Osteoblasts are specialized mesenchymal cells that synthesize bone matrix and coordinate the mineralization of the skeleton.

Osteoclasts are giant cells containing between 10 and 20 nuclei which are involved in remodeling of bone matrix.

Osteoconductivity is the property of a material to support bone tissue ingrowth.

Osteocytes are cells that lie within the substance of fully formed bone.

Osteogenicity means the extent to which something (a material, a cell, or a growth factor) allows bone formation.

Osteoinductivity is property of a material to stimulate osteoprogenitor cells to differentiate into osteoblasts.

Osteons are the basic units of compact bone, and are made of a hollow central canal (Haversian canal) surrounded by concentric bone layers named lamellae. Blood vessels and nerves run longitudinally through the Haversian canals.

Periosteum is a membrane that covers the outer surface of all bones, except at the articular surfaces.

Phenotypic markers are the observable characteristics of a cell resulting from the interaction of its genotype with the environment. For example, the production of cell specific proteins.

Regeneration is the process of renewal, restoration, and tissue growth.

Terminal differentiation is a state where a precursor cell formerly capable of cell division, permanently leaves the cell cycle, dismantles the cell cycle machinery, and often expresses a range of genes characteristic of the cell's final function (e.g., the presence of myosin and actin in a muscle contractile cell).

Traumatic injury is any injury that has the potential to cause prolonged disability or death.

Xenograft is the transplant of an organ, tissue, or cells to an individual of another species.

© Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Tissue engineering of the nervous system

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17.1 Learning objectives

After reading this chapter you will be able to:

- Appreciate how the regenerative potential of the mature peripheral nervous system (PNS) contrasts with the more limited capacity for regeneration in the mature central nervous system (CNS).
- Appreciate the critical gap length in peripheral nerves, and how different tissue engineering strategies are being developed to successfully bridge large nerve defects.
- Recognize there are different types and stages of spinal cord injury (SCI) and how this affects potential treatments.
- Understand that scar formation following injury to the adult CNS presents a key challenge for circuit repair and appreciate the importance of influencing the associated immune and inflammatory responses for successful regeneration.
- Appreciate that different therapeutic approaches are needed when treating traumatic versus degenerative brain pathologies, and note the unique nature of the optic nerve as a model for CNS regeneration.

When peripheral nerve segments were used as “bridges” between the medulla and spinal cord, axons from neurons at both these levels grew approximately 30 millimeters.

S. David and A. J. Aguayo, 1981

One man’s “magic” is another man’s engineering.

R. A Heinlein, 1973

17.2 Introduction

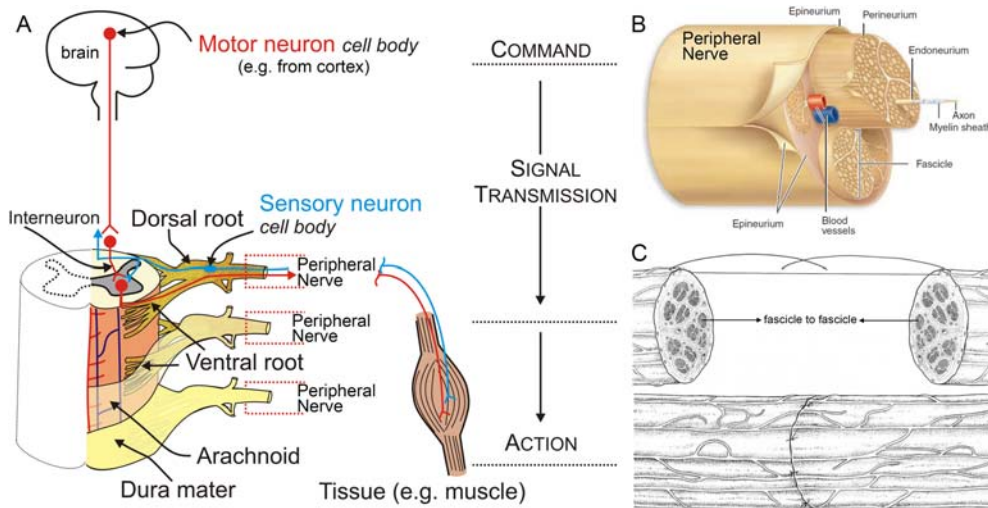
Diseases or injuries to the nervous system are devastating to the individual and have substantial societal implications and costs. The PNS and CNS—spinal cord, brain, and retina—have different molecular and cellular environments and, therefore, require specific tissue engineering repair strategies. For example, compared to the PNS, there is more limited plasticity and regrowth capacity in the CNS, due to inhibitory environmental factors that suppress growth as well as intrinsic changes to the regenerative capability of injured mature neurons. The primary focus of tissue engineering in the PNS or CNS is the regeneration and reconnection of severed axons that have been isolated from their targets. In the PNS, this often requires the implantation of artificial structures to bridge nerve defects in order to support and guide regenerating axons. Depending on the **neuropathological condition** under investigation, injectable matrices for the CNS can provide a substrate for axonal growth in the inhibitory CNS environment, and act as a delivery vehicle and/or provide a favorable environment for repair-supporting cells. Because of a long history of bridging studies in the PNS, this system is discussed first, followed by a description of tissue engineering strategies that are being developed for CNS repair.

17.3 Peripheral nerve

Peripheral nerve injuries (PNIs) can lead to lifetime loss of function and disfigurement even though a **peripheral nerve (PN)** is capable of a substantial amount of regeneration when crushed or severed. PN contains only the **axon** part of each contributing neuron and one could consider the peripheral nerve trunk as a protective structure for axons. The cell bodies of **sensory neurons** that give rise to PN axons are located in structures adjacent to the spinal cord (dorsal root ganglion (DRG)) or in cranial ganglia, while the cell bodies of **alpha motor neurons** that provide axons to innervate and drive skeletal muscle contraction are located within the CNS (brainstem or spinal cord gray matter). **Gamma motor neurons** are a different type of cell that also have their cell bodies in the spinal cord, but whose axon has a smaller diameter compared to alpha neurons. Gamma neurons do not innervate muscle fibers involved in contraction but contact sensory receptors called muscle spindles, thereby playing a critical role in monitoring and adjusting sensitivity to muscle length and stretch. Ideally, successful PN repair should involve all these neuronal classes. Once a PN defect is bridged, regenerating axons have demonstrated guidance over long distances within the degenerated distal nerve stump, along naturally occurring conduits called bands of Büngner (see **Bands of Büngner**).

17.3.1 Peripheral nerve anatomy

Peripheral spinal nerves (Fig. 17.1) are formed from dorsal or ventral roots of the spinal cord. Dorsal roots contain sensory (or afferent) axons of the somatic and **autonomic nervous systems** which transmit signals into the CNS from the periphery (skin, muscles, joints, etc.). Ventral roots contain motor (or efferent) axons which transmit motor signals from CNS-originating neurons out to muscles and glands. Fig. 17.1a shows schematically drawn cell bodies of sensory neurons (blue) and alpha motor neurons (red) and their corresponding nerve processes. Motor commands typically originate in the brain (cortex, midbrain, and brainstem) and contact lower motor neurons in the brainstem motor nuclei or ventral spinal cord, sometimes directly but more commonly via interneurons located within the gray matter. In addition, there are twelve **cranial nerves (CNs)**, the majority with nuclei located in the brainstem. CN I (the olfactory nerve) and CN II (optic nerve) are considered part of the CNS, while CN III–CN XII are in the PNS. Individual CNs can be purely sensory or motor or may contain both types of axons.

**FIGURE 17.1**

(a) Anatomical overview of the peripheral nerve and connections to the central nervous system. (b) Ultrastructure of the peripheral nerve, (c) Fascicle-to-fascicle alignment during microsurgical reattachment using sutures in a tensionless manner. (a) From Dalton, P.D., et al., 2008. *Tissue engineering of the nervous system*, In: Van Blitterswijk, C., et al. (Eds.), *Tissue Engineering*. Academic Press, pp. 611–647. (b) From Mescher, A.L., 2013. *Junqueira's Basic Histology*, 13th ed. McGraw-Hill. (c) From Lundborg, G., A 25-year perspective of peripheral nerve surgery: evolving neuroscientific concepts and clinical significance. *J Hand Surg* (0363-5023 (Print)).

PNs are discrete fibro-collagenous trunks (Fig. 17.1b) filled with sensory and motor axons and include Schwann cells, perineurial cells, and fibroblasts. Due to limb movements and the resulting tensile and compressive stresses, the outermost sheath, the epineurium, provides a protective structure to the axons. This fibro-collagenous epineurium binds individual axon-filled fascicles into one nerve trunk (Fig. 17.1b). Many (but not all) of the axons are myelinated by supporting glial cells called Schwann cells. Smaller diameter, nonmyelinated axons are embedded within invaginations of Schwann cells forming so-called “Remak bundles.”

17.3.2 Peripheral nerve injury

Regeneration of severed axons requires that the parent cell bodies survive the initial trauma. After injury to a PN, **retrograde degeneration** in the nerve stump occurs back to the node of Ranvier (see Section 17.14) that is located most proximal to the lesion. It is important to note that axonal injuries that are closer to the spinal cord or brainstem are more likely to cause retrograde neuronal cell death and are more complex than injuries that are located in more distal PN. After the initial PNI, the **proximal** nerve stump will swell, but undergoes minimal damage compared to axons in the **distal** stump. After a nerve injury, the distal axons degenerate by a process termed **Wallerian degeneration** (in both PNS and CNS, a sequence of active degenerative/reactive events in the distal axon); changes in PN include axonal cytoskeleton breakdown and shedding of myelin lipids by Schwann cells. Over several days, macrophages and Schwann cells clear **myelin** and other debris: a critical process that increases the potential for PN regeneration. Importantly, Schwann cells in the distal portion of the severed nerve proliferate and begin to secrete specific growth factors that stimulate axonal regrowth. After debris clearance, axonal sprouting and regeneration begins at the tip of the proximal stump and continues toward the distal stump.

Surgically, accurate realignment of the original fascicles is critical to increase the chances of functional recovery in microsurgical repair (Fig. 17.1c) and the ability to achieve “fascicle-to-fascicle” repair of large injury gaps is an important long-term goal for PN tissue engineering.

In the distal nerve stump, basal lamina tubules form Schwann cell filled bands of Büngner, which play a crucial role in successful guidance of regrowing axons to their original targets in PN regeneration.¹ Axonal growth in humans occurs at a rate of about 1 mm/day and appears to be more effective in younger people. Significant injuries closer to the CNS therefore can take many months to heal; not only must axons regenerate and reestablish the correct connections, but remyelination needs to occur and also maturation of synapses is required. This prolonged period of denervation has deleterious effects on the Schwann cells within the distal nerve stump and on target tissues, making it important that regeneration occurs as efficiently and as quickly as possible.

Anatomical nomenclature

Rostral or Anterior = Head or front end.

Caudal or Posterior = Tail or hind end.

Proximal = The (injured) part of the tissue closer to the neuron cell body.

Distal = The (injured) part of the tissue away from the neuron cell body.

Lateral = Away from the midline.

Medial = Toward the midline.

Dorsal = Back/top side.

Ventral = Belly/bottom side.

17.3.3 Autologous nerve grafts (autograft)

PNI most commonly results from blunt trauma such as crush injury or from penetrating injuries caused by sharp instruments, but it is also associated with fractures and fracture dislocations. Crush injuries are therefore more common than nerve transections. When nerve stumps are unable to be surgically reattached using sutures in a tensionless manner, a bridging section of an implanted autologous nerve is often used, and two end-to-end sutures are performed. The damaged tissue is replaced by a nerve taken from another (less critical) site, for example, the purely sensory sural nerve.² However, this also results in some **donor site morbidity** and loss of sensation to the lateral heel/foot region. The inability to match the fascicular pattern of the donor nerve to that of the damaged host nerve may lead to some degree of misrouting of regenerating axons in which sensory axons may enter and follow the original trajectory previously occupied by motor axons (and vice versa). Despite great care being taken during microsurgical repair, axonal misdirection is a frequent problem. Furthermore, donor nerves are often of small caliber and limited in number. Although the PN autograft is regarded as the “gold standard” to which all alternative therapies are compared, the above-mentioned problems have driven the search for tissue engineering alternatives to this treatment.

17.3.4 Use of nerve guides (tubes) in the lesioned PNS

An off-the-shelf alternative for injury-induced gaps of PN (typically less than 10 mm but up to 70 mm) are **nerve guides** which function by acting as a conduit between the severed proximal and

distal nerve stumps. Hollow tubes (nerve guides or nerve conduits) have successful outcomes that are comparable to the autograft in certain clinical situations. Fig. 17.2 schematically shows the general sequence of regeneration. After implantation, fibrin from damaged blood vessels and cytokines (including neurotrophic factors) primarily generated by the Schwann cells builds up within the nerve guide (Fig. 17.2a). Within 7 days, an oriented fibrin scaffold is formed, and cells from the perineurium penetrate into the nerve guide, while axonal debris is removed by Schwann cells and macrophages in the distal stump (Fig. 17.2b). From 7 to 14 days, migrating endothelial cells, Schwann cells, and regenerating axons penetrate into the nerve guide using the newly formed fibrin scaffold as a substrate (Fig. 17.2c). Between 14 and 56 days, Schwann cells remyelinate the regenerating larger diameter axons that exit the nerve guide, directed by the Bands of Büngner in the distal nerve stump to their targets.

Currently, there are several nerve guides on the medical device market, as shown in Table 17.1. Their performance is reviewed in depth by Deumens et al.,². All of these hollow nerve guides are biodegradable and provide a protective semipermeable membrane which separates the injury site from surrounding tissues and through which nerve regeneration and tissue repair are supported. Current research focuses on filling the lumen of nerve guides with matrices, scaffolds, cells, and/or drug delivering therapies to increase the efficiency of tissue repair.^{3,4}

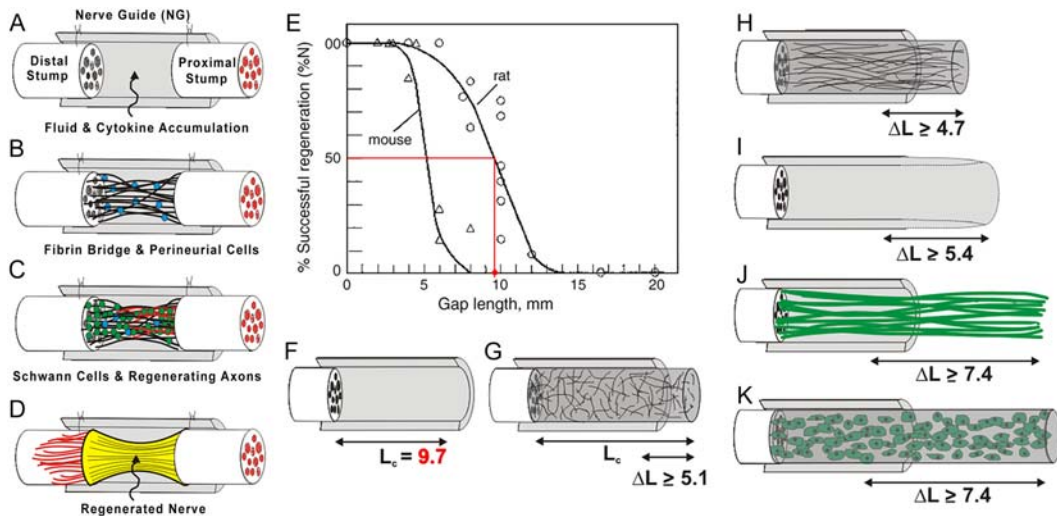


FIGURE 17.2

Progression of regeneration within a nerve guide: (a) hours, (b) days, (c) weeks, and (d) months after injury. (e) Critical gap defect (L_c) and regenerative capability. An empty nerve guide (f) increases (g) its L_c value with (h) oriented matrices, (i) degrading polymers, (j) fiber inclusion, or (k) Schwann cell transplantation. (e) From Zhang, M., Yannas, I.V., 2005. *Peripheral nerve regeneration*. *Adv Biochem Eng Biotechnol.* 94, 67–89.

Bands of Büngner

Although the critical gap length (L_C) for humans is approximately 30 mm, once the regenerating axons bridge this defect, there is a natural scaffold prepared in the distal segment of the peripheral nerve. Axons will travel distances well above the critical gap length along Schwann cell—containing tubular basal lamina, which are termed “Bands of Büngner” after the German neurologist Otto von Büngner (1858–1905) and are often described as “Schwann cell columns.” After PNI, the cytoskeleton of the severed axon is degraded and Schwann cells, which were previously wrapped around the axons, disperse with their myelin sheath and clear debris, adopting a repair phenotype. The Schwann cells partly clear the myelin while increasing numbers of macrophages in the distal stump

complete the task. Throughout such Wallerian degeneration, the basal lamina tubes that support these cells remain and act as specific tracts that channel the regenerating axons to the final targets.

With the development and optimization of microsurgical techniques for peripheral nerve repair, the success of end-to-end suturing greatly depends on the alignment of the fascicles. Therefore, the penetration of a sensory axon down a basal lamina tube previously filled with an axon from a motor neuron (and vice versa) is an ineffective (but frequent) result. Nevertheless, the bands of Büngner are the naturally occurring substrate for regeneration over long distances distal to the PN injury.

17.3.5 Critical gap length

A **critical gap length** (L_C)⁵ is an indication of the limits of PN regeneration using nerve guides. It is the distance between transected nerves where the regeneration success is approximately 50% and varies depending on species.⁶ When the transected PN length approaches this experimentally determined length, the chances of successful regeneration within nerve guides are diminished. Zhang and Yannas have shown the frequency of successful regeneration (%N) within rat and murine models using silicone nerve guides and L_C (when %N = 50) has been determined to be 9.7 ± 1.8 mm for rats and 5.4 ± 1.0 mm for mice.^{5,7} The introduction of matrices, fibers, and cells within nerve guides likely increases the L_C to varying degrees, which is an important measure of therapeutic success. The change in length (ΔL) due to a therapy is the difference between the L_C of the experimental device and the L_C of the control group. The introduction of a therapy will generally increase the L_C ; Fig. 17.2 shows schematics based upon this approach of assessing additional therapies. Using the L_C normalization of therapies in the PNS, the therapies that significantly increased ΔL were analyzed and regenerative theories put forward.⁷

17.3.6 Nerve guides as supports for regeneration strategies

Nerve guides are often filled with a therapeutic agent to improve the L_C . Matrices and scaffolds for the PN aim to mimic an ECM environment that promotes increased regeneration after injury, while cellular grafts and neurotropic factors provide an improved **chemotactic** environment after injury.

Matrices

Currently, no ideal matrix promotes the regeneration in a nerve guide that is better than the level achieved using a PN autograft³; however, the L_C generally increases for nerve guides when they contain matrices within the lumen. Effective matrices have similar mechanical properties in that they are all soft, viscoelastic hydrogels with high water content; however, they cannot be too concentrated (or dense) as

this would lead to obstruction of cell and axonal penetration. While matrices are formulated into **bio-inks**⁸ for biofabrication approaches, these require the capacity to be translated to the clinic. It is important to mention that when the nerve guide is sutured into position, a fibrin bridge spontaneously forms due to the secretion of factors from the proximal and distal stumps. This natural process has been taken advantage of in order to engineer matrices that fill the lumen of nerve guides.

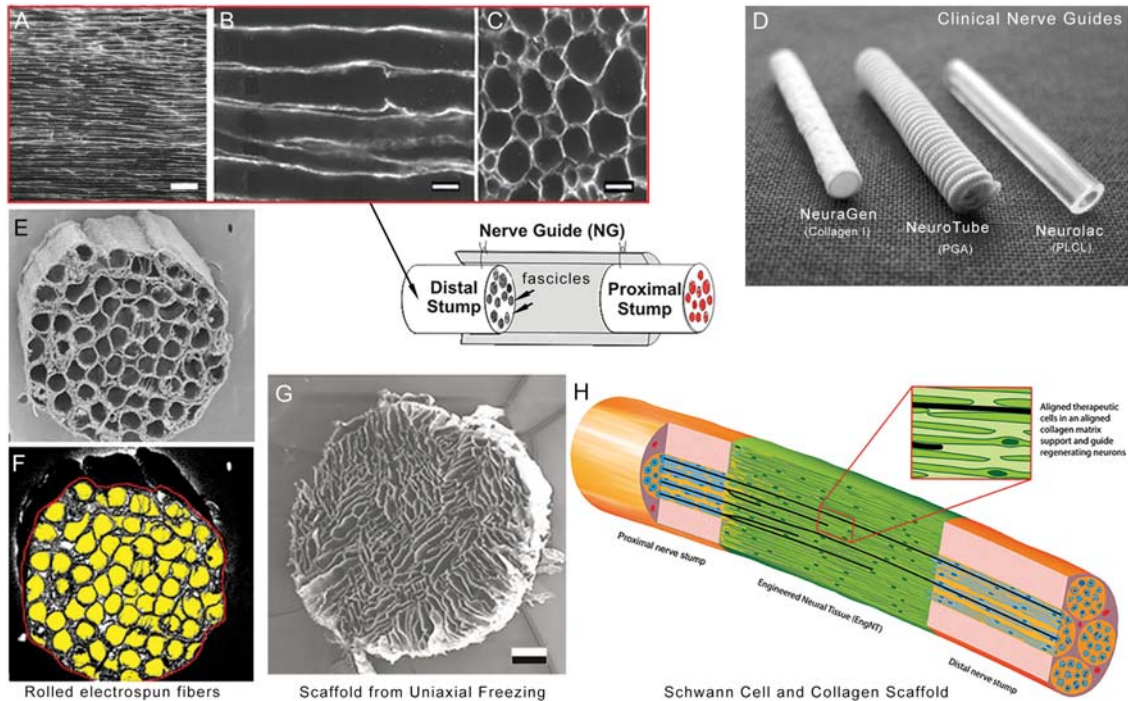
Oriented matrices

In vivo experiments with oriented matrices have a higher L_C value than nonoriented matrices of the same material (Fig. 17.2h). This was shown with fibrin and collagen, which can be aligned with a magnetic field during gelation due to the fact they are diamagnetic macromolecules.⁹ Oriented substratum and techniques to form such structures on a relevant scale are therefore important issues in neural TE. Axons will regenerate significant distances along Schwann cell—containing basal lamina tubes or similar structures (see **Bands of Büngner**).

Scaffolds

The endoneurium of the PN consists of the interstices lying between the longitudinally oriented basal lamina tubes and the perineurium and is rich in highly aligned collagen fibrils (Fig. 17.3a and b). It is widely anticipated that similar channel-like morphologies would be ideal in the supporting directed growth of neurites. While a nerve guide (Fig. 17.3c) contains the severed stumps of the PN, the nerve itself has a structure based on nerve fascicles containing myelinated and nonmyelinated peripheral axons (Fig. 17.3d). Jeffries and Wang¹⁰ produced channeled nerve guides by combining oriented electrospun fiber collection with channel templates, which could be rolled up to produce the channel structures shown in Fig. 17.3e–f. One simple technique to induce orientation involves the controlled growth of ice crystals through the hydrogel matrix to impart similar guidance structures (Fig. 17.3g). Such approaches are applicable to a range of hydrogels, essentially converting oriented materials into scaffolds with guidance properties.^{11,12} The NeuroMaix nerve guide developed by Matricel GmbH is in clinical trials and is based on this uniaxial freezing approach.¹³ Simple molding and construct dissolution is another approach to create guidance channels, as rendered in Fig. 17.3h.¹⁴ For such channels, surface modification of synthetic polymers with peptide sequences such as RGDS (Arg-Gly-Asp-Ser), YIGSR (Tyr-Ile-Gly-Ser-Arg), and/or IKVAV (Ile-Lys-Val-Ala-Val) significantly increases neurite growth in vitro. Scaffolds made using biologically derived polymers such as collagen inherently contain such cues that aid in the guidance of neurites.¹⁵

Multiple studies using submicron diameter fibers will also guide growth cones and neurites. Fibers are attractive guidance substrates as they can be bundled together and used as oriented scaffolds (Fig. 17.3e–f). Interestingly, when oriented collagen fibers are embedded within a hydrogel, the Schwann cell will significantly stretch more than one placed in that hydrogel environment alone (Fig. 17.3h).¹⁶ Using oriented electrospun sheets within nerve guides, a more challenging 14 mm nerve gap could be repaired.¹⁷ There remains extensive research to perform on scaffold manufacturing technologies (see Chapter 11 **Scaffold design and fabrication**) for PNI, including structures and morphologies that better mimic fascicles. Optimal structures likely will replicate the natural fascicle structure and Fig. 17.3 highlights how scaffold design may aim to replicate this morphology.

**FIGURE 17.3**

Strategies to mimic the peripheral nerve ECM architecture. (a–c) Natural ECM of the PN, (d) Clinical nerve guides, (e and f) Scaffold and channels fabricated with an electrospinning approach, (g) an oriented scaffold made using a freezing technique, (h) aligned collagen matrix. (a–c) From Ceci et al., 2014. Axon-Schwann cell interactions during peripheral nerve regeneration in zebrafish larvae. *Neural Dev* 1749–8104. (d) From Tian et al., 2015. Strategies for regeneration of components of nervous system: scaffolds, cells and biomolecules. (e and f) From Jeffries and Wang, 2012. Biomimetic micropatterned multi-channel nerve guides by templated electrospinning. *Biotechnol Bioeng* 109 (6), 1571–1582. (g) From Riblett et al., 2012. Ice-templated scaffolds with microridged pores direct DRG neurite growth. (h) From Phillips, 2021. “EngNT”—Engineering live neural tissue for nerve replacement.

Acellular allografts

As an alternative to autologous tissues and nerve guides, nonautologous sources have also been explored for bridging of PN lesions. For this purpose, both **allogenic** (same species) and even **xenogeneic** (different species) tissues have been considered. Although such allogenic and xenogeneic alternatives may be in plentiful supply, they present several risk factors including immunogenic mismatching as well as the possible transmission of disease. Immune suppression may be used to prevent graft rejection in such circumstances, but long-term immune suppression is not the ideal solution.

The treatment of allogenic nerves involves freeze-thawing of donor tissue to reduce immunogenicity by effectively killing all cellular components (e.g., SC, perineurial cells, and fibroblasts) while leaving the ECM (see Chapter 5 **Extracellular matrix as a bioscaffold for tissue engineering**), including SC basal laminae and endoneurial collagen fibrils, largely intact. Such cell-free PN grafts have been reported to

be far less immunogenic.¹⁸ Grafts such as the FDA-approved Avance product (Table 17.1) promote successful regeneration across PN deficits of up to 30 mm.¹⁹

Schwann cell grafts

When cultured Schwann cells are included within nerve guides, large shift lengths (ΔL) result and significant gap lengths may be bridged (Fig. 17.2k). For example, the morphological and phenotypic characteristics of the bands of Büngner have served as inspiration for a biofabricated Schwann cell–laden implantable microtissue. These structures successfully increased the rate of axonal extension in vitro from primary rat spinal motor neurons and rat DRG sensory neurons.²⁰ Schwann cells can also be suspended within a matrix or seeded within decellularized PN allografts.¹⁸ The challenge with using Schwann cell transplants is that their regenerative phenotype is usually transient.²¹ For cellular nerve graft or cell/biomatrix hybrid structures, the long-term supply of **neurotrophic factors** to promote axonal regrowth may be achieved by the Schwann cells themselves, or specific factors can be obtained by targeted genetic modification of Schwann cells using viral vectors in vivo or ex vivo.¹⁸ Depending on the type of growth factor that is introduced into grafted Schwann cells, differential effects have been reported on graft morphology, the number and type of regenerating sensory and motor axons, the extent of remyelination, and the degree of functional recovery including locomotion.^{18,22–24} Schwann cell transplantation has been a primary focus for cell therapies in the treatment of PNI, although the source of the Schwann cells is another factor that needs to be addressed. Any eventual clinical solution for bridging large injury gaps will have to compete with the relatively low-cost alternative of the autograft, nerve guides, and decellularized allografts. In addition to Schwann cells, other cell types including stem cells (mesenchymal or adipose-derived) or bone marrow cells (including stromal or mononuclear cells) have been paired with a nerve prosthesis to repair **critical-sized defects** in peripheral nerves.²⁵

Table 17.1 Commercially available and FDA approved nerve conduits.

Product name	Composition	Diameter	Length	Degradation time	Manufacturer
Neurotube	Poly(glycolic acid)	2–8 mm	4 cm	3 months	Synovis Micro Companies Alliance, Birmingham, AL
NeuroMatrix Neuroflex Neuromend	Type 1 collagen	2–6 mm	2.5 cm	7 months	Collagen Matrix Inc., Franklin Lakes, NJ
Neurolac	Poly(lactide-co-caprolactone)	1.5–10 mm	3 cm	16 months	Polyganics BV, Netherlands
NeuraGen	Type 1 collagen	2–7 mm	2 cm	4 years	Integra Neuroscience, Plainsboro, NJ
SaluBridge	Salubria (poly (vinyl alcohol) hydrogel)	2–10 mm	6.35 cm	No degradation	SaluMedica LLC, Atlanta, GA
Advance	Decellularized human nerve allograft	1–5 mm	7 cm	No data, but expected to resorb	Axogen Corp., Alachua, FL

Adapted from Schlosshauer, B. et al., 2006. Synthetic nerve guide implants in humans: a comprehensive survey. *Neurosurgery* 59 (4), 740–747; discussion 747–748.

Neurotrophic factors

After implantation, the lumen of nerve guides shows elevated levels of cytokines and trophic factors. Cytokines are a broad family of small proteins secreted by cells that have a specific effect on cell–cell interactions and communication (see Chapter 4 **Cellular signaling**). An important neuropoietic cytokine released by glial cells as a response to injury is ciliary neurotrophic factor (CNTF) because it promotes cell survival or differentiation and can influence regenerative capacity. Other neurotrophic factors also play an important role in assisting neuron survival after severing of the axon (also called **axotomy**). These factors include neurotrophins and fibroblast growth factors and originate from Schwann cells in both the proximal and distal stumps of the severed nerve.⁹ The neurotrophins include nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and brain-derived neurotrophic factor (BDNF). These molecules signal through tyrosine kinase (Trk) receptors in the cell membrane in conjunction with low-affinity receptors such as the low affinity nerve growth factor receptor (NGFr/p75). After injury, NGF is elevated in the distal nerve stump and acts through TrkA on sprouting nerve fibers to assist in the survival and outgrowth of a proportion of sensory neurons. Other sensory neurons express different receptors and are supported by NT-3¹⁸ or by other factors such as glial-derived neurotrophic factor (GDNF). BDNF and NT-3, respectively, signal through TrkB and TrkC receptors that are present on alpha motor axons and increase neuron viability and sprouting after axotomy. Increased brainstem and spinal cord alpha-motor neuron survival has been reported using exogenously supplied GDNF and BDNF.²² Nonviral neuronal targeted delivery of genes encoding for BDNF has also proven to be efficient in promoting neuroprotection and regeneration after an injury.²⁶ An *in vivo* study combining GDNF and NGF in delayed release profiles from nerve guides demonstrated a synergistic effect and significantly increased axonal regeneration above either GF alone or controls.^{18,22}

There are several approaches to deliver neurotrophic factors in biocompatible nerve guides. The growth factor can be embedded within the nerve guide, homogeneously, or specifically bound to the luminal wall, effectively directing the release into the lumen.²⁶ The matrix in the lumen of the nerve guide is also an excellent substrate for binding growth factors; either simply adsorbed to the substrate surface, or as part of a noncovalent controlled release system. Heparin-binding growth factors (e.g., NGF, NT-3) can also be locally bound to the matrices allowing progressive delivery to regenerating axons.²³ The potential permutations and concentrations of neurotrophic factors are many; cocktails of different neurotrophic factors may result in enhanced neuron survival and gradients of such factors lead to directed axonal or cell growth (see Chapter 12 **Controlled release strategies in tissue engineering**). However, it may be more physiologically relevant to establish defined gradients of growth factors along the damaged nerve to prevent the potential “candy store” effect of axonal growth slowing or even stopping at the site of elevated factor delivery.

17.3.7 Biofabricated nerve guides

Biofabrication describes the use of 3D printing or other digital-based manufacturing approaches within tissue engineering (see Chapter 12 **Controlled release strategies in tissue engineering**). There are many different types of 3D printing technologies and many have been used to treat PNI.²⁷ The logical approach is to fabricate nerve guides with thin walls or in a specialized purpose. Injuries at the bifurcation (splitting) of the PN create challenges in standard nerve guide repair (Fig. 17.4). In

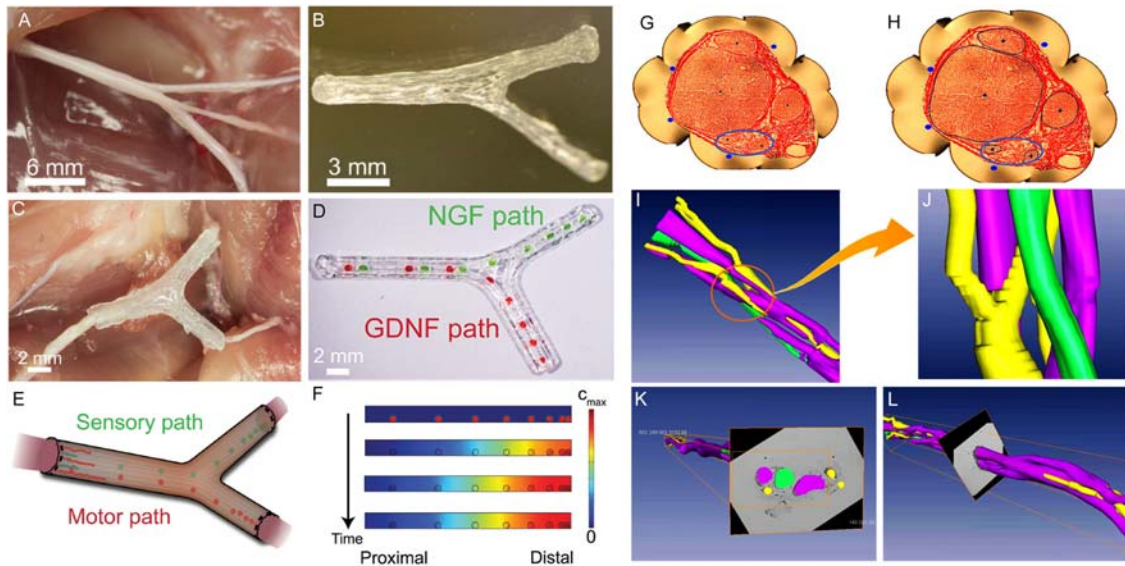


FIGURE 17.4

Examples of 3D printing and 3D rendering software for nerve guides. 3D printed construct (a–f) that allows splitting of the nerve guide. (g and h) Histological sections of human nerve allow the recreation of the tortuous 3D fascicles shown in (i–l). (a–f) From Johnson *et al.*, 2015. 3D printed anatomical nerve regeneration pathways. *Adv Funct Mater* 25 (39), 6205–6217. (g–l) From Zhong *et al.*, 2015. Three-dimensional reconstruction of peripheral nerve internal fascicular groups. *Sci Rep* 5, 17168.

this example, the bifurcated nerve was scanned, and a digital model developed that could be 3D printed using extrusion processing.²⁸ Furthermore, NGF and GDNF gradients were approximated using precise placement of matrices loaded with the appropriate growth factor (Fig. 17.4d–f). Fig. 17.4a–f highlights both the promise of biofabrication and challenges currently faced, with improvements in resolution levels still requiring the production of thin-walled structures with minimal profile differences such as shown in Fig. 17.4c.

Currently, the resolution of digital models and the ability to rapidly process data into customizable nerve guides²⁹ outpaces the ability to print the required resolutions. A common feature of fabricated nerve guides with internal structures is that there is low porosity within the nerve guide. For example, Fig. 17.4g–l are digital reconstructions of human nerve fascicles, reconstructed from histological sections. The tortuosity and thin structures that compose these guiding fascicles highlight how simple it is for a nonguided axon to grow into the incorrect fascicle at the distal end. Other imaging approaches also can identify fascicles, although these structures are too fine to be accurately printed.³⁰ With further improvements in 3D printing resolution, such fascicular structures could be accurately fabricated within nerve guides to increase the critical gap length.

17.3.8 Bioprinting for the PNS

Incorporating viable cells within biomaterials throughout the printing process with a technique known as **bioprinting** can be used to build cell-laden scaffolds that aim to mimic the structure and function of

neural tissue. Human gingiva–derived mesenchymal stem cells (MSCs) were 3D bioprinted into nerve constructs and have been shown to bridge segmental defects in rat facial nerves with successful functional recovery *in vivo*.³¹ Gingiva-derived MSCs were chosen not only due to their capacity to self-renew and differentiate into functional glial and neuronal cells (feature common to all MSCs) but also due to their potential to be induced to neural progenitor-like cells (NPCs) with improved therapeutic effects on PN regeneration. Successful bioprinting of rat Schwann cells by extruding cell-laden fibrinogen into a thrombin solution was the first example of combining biomaterials to bioprint neural cells.³² Other approaches to bioprint these cells have also been explored for various applications in the CNS (see Sections 17.3.7 and 17.4.4).

17.3.9 PNS summary

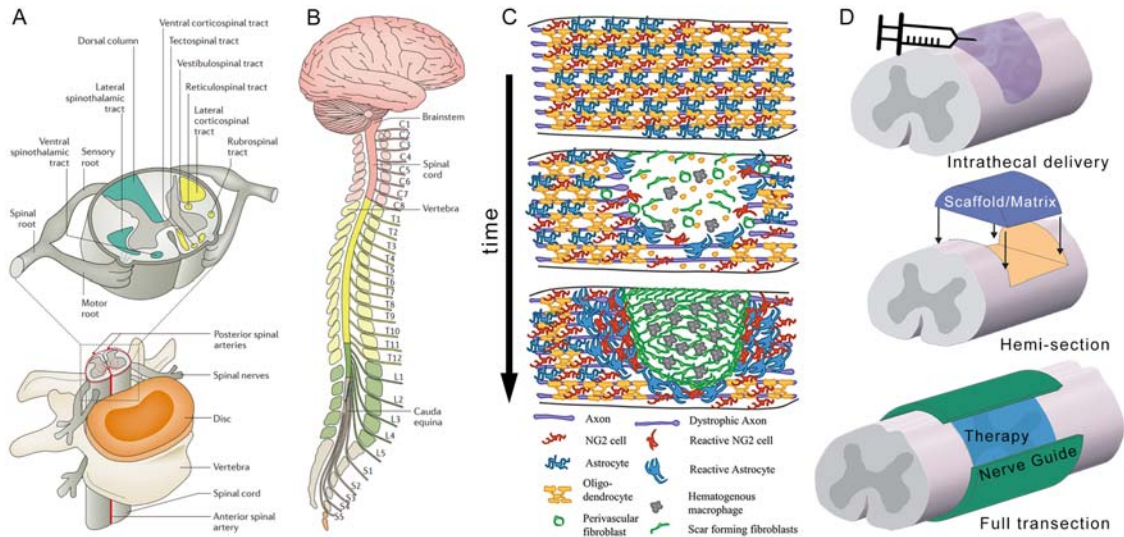
Small defects to the PN may be repaired by suturing nerve stumps together, but larger nerve gaps require additional approaches in order to bridge proximal and distal ends. The standard approach to connect the nerve stumps is autograft implantation, which endorses axon regrowth; however, besides causing a lesion at the donor site, it is rare that axon regrowth results in full reinnervation and return of sensory and motor functions to an injured patient. Alternatively, a biomaterials-based approach involves *in vivo* implantation of artificial scaffolds and substrates that will bridge the defect and guide axons with innate ability to regenerate. Current tissue engineering strategies to treat PNI with nerve guides range from Schwann cell or stem cell grafts, bundled fibers or channels, or oriented scaffolds/matrices placed within nerve guides. These approaches have been further paired with gene therapy or biochemically stimulated hydrogel filling in order to create a gradient that further promotes axonal regeneration following PNI.

17.4 CNS: spinal cord

Numerous animal trials to treat SCI have achieved some success although such approaches have yet to be successfully translated to the clinic. Damage to the spinal cord results in the immediate death of neuronal cells and disrupts the blood supply to the injury area. This in turn compromises the neuronal cell bodies present in the spinal cord, which presents a major difference to damage of the PNS, where only axons are initially affected. Furthermore, a **spinal injury scar** develops that impedes potential effective endogenous or treatment-driven regeneration. This scar consists mainly of reactive astrocytes, fibroblasts, pericytes, ECM, and myelin debris. These scar cells can express axonal growth inhibitory molecules such as chondroitin sulfate proteoglycans and semaphorins and so form a chemical and physical barrier for axon regeneration.^{9,33} There are also immune/inflammatory changes and extensive neuronal and glial degeneration as a secondary response to the initial injury that further reduces the prospect for nervous tissue repair and functional recovery.

17.4.1 Summary of anatomy and injury response

The core of the spinal cord consists of a butterfly-shaped gray matter containing neuronal cells, support cells (termed **glia**), and numerous blood vessels (Fig. 17.5a). The white matter surrounding this structure contains mainly axons and **oligodendrocytes** (a type of glial cell that provides myelin insulation to CNS axons), astrocytes, microglia (immune cells), and relatively fewer blood vessels. The spinal cord is wrapped within multimembrane layers (dura mater, arachnoid, and pia matter) (Fig. 17.5b) that form the

**FIGURE 17.5**

(a) Anatomy of the spinal cord including the surrounding anatomy of the spinal meninges (b), (c) schematic of a spinal cord scar formation, (d) intrathecal, hemisection, and full transection models for researching SCI models. (a and b) From Ahuja et al., 2017. *Traumatic spinal cord injury*. (c) From Hackett and Lee, 2016. *Understanding the NG2 glial scar after spinal cord injury*. *Front Neurol* 7(199).

intrathecal space in which the cerebrospinal fluid (CSF) circulates. SCI results in disruption of both sensory and motor axons, and this lack of anatomical and functional continuity in the spinal cord has devastating consequences for injured patients. The initial injury response following bruising or transection of the spinal cord involves a primary event in which cells and tissue are crushed or cut by the injury and cells undergo **necrosis**. The primary injury initiates secondary events that include altered local blood flow, activation of immune cells and influx of macrophages, ionic imbalance including raised Ca^{2+} levels, release of inflammatory cytokines, oxidative stress, and fluid build-up within the lesion site. Macrophage/microglia-mediated removal of damaged nervous tissue results in the formation of fluid-filled cysts (Fig. 17.5c). Secondary inflammatory events cause neurons in the gray matter, and oligodendrocytes in the white matter, to undergo **apoptosis**. Death of oligodendrocytes and subsequent loss of the myelin sheath interferes with the speed and reliability of electrical conduction in any remaining intact axons, significantly contributing to a sustained and long-lasting loss of function. Successful spinal cord repair requires all these issues to be targeted. This has proved to be a complex and difficult task.

The tissue engineering challenge to overcome spinal cord scarring

CNS white matter, which contains a range of myelin and myelin-associated axonal growth inhibitors, restricts any substantial axonal regenerative response in the adult spinal cord, regardless of injury. In a seminal paper by David and Aguayo in 1981, the transplantation of peripheral nerves into the spinal cord showed that it was the physical/molecular environment that prevented regeneration and not necessarily a factor that is intrinsic to CNS neurons.³⁴ The use of scaffolds and matrices in the spinal cord provides an extracellular environment for regrowth and repair-supporting molecules and/or cells

and/or provides a terrain for severed ascending (sensory) or descending (motor) axons to grow across the site of injury. Acellular substrates (scaffolds/matrices) may elicit an axonal regeneration response³⁵ but typically are less effective than cellular transplants.

After SCI, a physical and chemical barrier (fibro-adhesive and glial scar) forms at the impact site which protects adjacent, spared CNS tissues from further damage by infiltrating monocytes but also limits the growth of new axonal sprouts. This scar consists mainly of reactive astrocytes, subtypes of pericytes and meningeal fibroblasts, which express extracellular axonal growth inhibitory molecules such as chondroitin sulfate proteoglycans and semaphorins. It has become clear that manipulation of this barrier will be an essential component of a successful repair strategy for the injured spinal cord. Such manipulation of the scar may include the following: (1) decreasing the inhibitory nature of the scar, (2) preventing axons from recognizing inhibitory molecules, and (3) enhancing the intrinsic growth ability of axons. The permissiveness of the scar for growing axons can be increased by blocking receptor–ligand binding. This reduces the synthesis of inhibitors or degrades biologically active components of the inhibitors, for instance, using the chondroitinase enzyme. By reducing the expression of growth inhibitors, cells can to some extent recover their ability to form and extend nerve processes, including axons. Examples of current approaches to promote regrowth include cell therapies and introducing inhibition antagonists,³⁶ the latter already the subject of clinical trials.³⁷

In the laboratory, several experimental approaches resulted in axonal regrowth through and beyond the scar, but success was usually obtained using injuries that could be considered as “smaller size” injuries, i.e., with less scar tissue formation. The barrier of scarring can be overcome by implantation of olfactory ensheathing glia (OEG) into the scar-forming environment,^{38,39} increasing the levels of neurotrophic factors in the nervous tissue using osmotic mini-pumps^{40,41} or the use of a modified form of the chondroitinase enzyme.^{42,43} Understanding mechanisms of reduced cyst formation and astrocytic-fibro-adhesive scarring associated with tissue engineering scaffolds and matrices is a fundamental area of investigation.^{44–46} Combining this knowledge is essential to develop scaffold- and matrix-based regenerative strategies that aim to bridge the lesion that results from SCI.

17.4.2 SCI models

There are numerous experimental animal models of SCI, mostly involving rodents (Fig. 17.5d). Different SCI models are best suited for the measurement of tissue sparing (contusion/compression), axonal regeneration (complete transection), or sprouting of intact fibers (hemisection). When using these models, it should always be borne in mind that the human spinal cord is comparatively very much greater in length and with some key differences in intrinsic organization,⁴⁷ and with chronic responses to injury that impede sensorimotor recovery.^{48,49}

Contusion animal model

Most human SCI is the result of a compression or **contusion injury**, and usually leads to the formation of cystic cavities as schematically shown in Fig. 17.5c. Experimentally, such injuries are modeled either by transient compression of the spinal cord using a weight drop method or a compression clip, or by displacement of the spinal cord using an inflatable device (e.g., the Fogarty inflatable balloon). With contusion/compression injuries, analysis of the axonal regeneration response is complex due to the

presence of spared axons and their collateral sprouts. To repair the contused/compressed spinal cord, cells or materials can be injected directly into, or next to, the contusion cavity, in small volumes. These injections into the lesion cavity may likely elicit a neuroprotective effect and/or an axonal regeneration response which could support some degree of functional recovery.

Hemisection model

Some instances of human SCI result from a laceration with disruption of the dura mater. This injury is mimicked in the laboratory using a surgical microknife or microscissors as depicted in 17.5D. Although with a partial (hemi) section the loss of tissue and function is limited, over time injury-induced secondary tissue loss may add to the overall injury zone. With a **hemisection SCI model**, as with a contusion/compression injury, the presence of spared axons that may form axon collaterals makes a definitive analysis of any transplant-induced regeneration response difficult. To repair the spinal cord following a hemisection, a bridging transplant can be placed in the lesion site. Examples of bridging transplants include preformed scaffolds with or without cell suspensions/in situ hydrogels⁴² and PN segments.⁴¹ While the grafted cells/tissues/scaffolds may elicit axonal growth in and across the hemisection, responding axons usually fail to exit the transplant without an additional intervention that facilitates their growth across the transplant–spinal cord interface. Examples of such additional interventions are increasing neurotrophic levels using osmotic minipumps or direct intraparenchymal injections (see Section 17.9) or introducing modified chondroitinase.⁴²

Full transection models

Full transection experiments (Fig. 17.5d) are not common due to surgical complications such as instability of the spinal column and the difficulty in animal care. However, this type of lesion, while less clinically relevant, can provide absolute evidence of true axonal regeneration. While hollow poly(acrylonitrile-co-vinyl chloride) nerve guides have been used for full transection (see Section 17.9), other investigations have used soft, flexible hydrogel nerve guides in the fully transected cord and demonstrate improved regenerative capacity.^{50,51}

Intrathecal delivery

The many blood vessels and capillaries that pass through the CNS (the gray matter is particularly highly vascularized) have ordered endothelial cells, tight junction molecules, and astrocytic end-feet that between them form the **blood–brain barrier (BBB)** and blood–spinal cord barrier. These blood–CNS barriers ensure that the two environments can be separated and many proteins and other molecules are not able to freely pass across them unless facilitated by, for example, nanodelivery systems.⁵² One way to deliver drugs to the CNS without having to cross the BBB is by lumbar puncture. An alternative way to deliver drugs to the brain is to inject fast-gelling adhering matrices with the drug into the space between the arachnoid and pia mater (intrathecal space) where the CSF flows.

A thermo-reversible hydrogel matrix blend of hyaluronic acid and methyl cellulose, called HAMC, was injected in the intrathecal space to successfully deliver modulatory drugs. The injectable and intrathecal delivery approach has flexibility in that cells or drugs can be delivered to modulate inflammation and limit the extent of injury.⁴⁴

Gender and age

Gender differences may affect the cascade of degenerative processes and functional outcomes after traumatic and ischemic CNS injuries, although there is some controversy.⁵⁶ While some studies argue that the presence of estrogens and progesterone likely plays an important role in the greater neuroprotection sometimes reported in females versus males after injury, the underlying biological mechanisms are not fully understood. Alternatively, other studies question the involvement of female sex hormones in outcome in specific SCI models and claim absence of gender differences. The importance of age in the pathophysiology of CNS and PNS injuries and in the ability to recover is well established. However, animal models do not always reflect these gender and age differences, and it may well be that for a particular type of injury different animal models should be developed that represent such differences.

Species selection for SCI models

The selection of the species for SCI studies is an important issue. Tissue engineering strategies have focused on the mammalian model, while invertebrates and nonmammalian vertebrates are aimed at understanding fundamental biochemical and molecular processes associated with cell survival and regeneration. The pathology of rat SCI is fairly similar to that in humans, although important differences have been discerned, including reduced glial scarring, inflammation and demyelination, increased Schwann cell infiltration, and protracted Wallerian degeneration in humans.⁴⁹ Mice are also commonly used and have the advantage that numerous genetically engineered strains are available. A notable difference between mouse and human SCI is that cysts usually do not form in mice. Note also that there are important strain-specific differences in how animals respond to experimental spinal cord trauma.

Larger species such as pigs, dogs, cats, and nonhuman primates are also used in SCI studies, with the latter being most relevant to the human condition. To assess the efficacy of specific approaches to repair the human spinal cord, it is increasingly accepted that the development and use of nonhuman primate models such as monkeys is advisable. However, in addition to ethical issues, the maintenance of the injured primate is extremely labor intensive. Alternatively, cats can also be used to model SCI because they offer important advantages over other models in that their spinal cord physiology is well described, and these animals can be trained relatively easily. Treadmill training of cats allows the animal to regain their ability to bear weight after spinal injury, whereas untrained animals show limited locomotor recovery. This difference in behavior when comparing untrained versus trained cats becomes a disadvantage to this animal model for SCI because experimental results are not representative of the proposed therapy. Locomotion recovery in cats after injury is strongly influenced by their **central pattern generator** in the spinal cord, enabling recovery of reflex stepping on a treadmill. The pattern generator in humans appears to be comparatively less effective after SCI.

17.4.3 Cell transplantation

Cell transplants into the spinal cord aim to encourage injured axons to grow through areas of damage toward and into their target areas, and/or as a means to deliver repair-supporting molecules. Various cell types have been tested including Schwann cells, OEG, and stem cells derived from either embryonic or adult tissues.

Schwann cells

Schwann cells can produce a variety of growth-promoting molecules that provide a growth substrate for injured CNS axons. In the injured spinal cord, Schwann cell transplants elicit axonal regeneration (see [Section 17.9](#)).^{38,54,57} Schwann cells also form myelin sheaths around CNS axons allowing signal conduction in regenerated CNS axons. In addition to axonal regeneration, an intraspinal Schwann cell graft promotes neuroprotection thereby reducing the additional loss of nervous tissue due to secondary events.³⁸ Schwann cells grafted into the lesioned CNS show little or no tendency to migrate and integrate with the host tissues due to a mutual repulsion exhibited between Schwann cells and CNS-derived astrocytes, as demonstrated in vitro.³³ Schwann cell transplants in the cystic cavity combined with maintaining an increased level of cyclic adenosine monophosphate result in improved functional recovery.⁵⁸

Olfactory ensheathing glia

The environment of the adult mammalian brain and spinal cord is generally nonpermissive for regrowth; however, there is one structure in the mature CNS where axonal growth does occur: the olfactory bulb. Throughout adulthood, newly formed olfactory neurons in the upper nasal cavity generate axons that grow through the cribriform plate into the olfactory bulb to make their appropriate connections in the brain. This particular growth response occurs because of the presence of OEG, which helps to shield the growing axons from nonpermissive CNS tissue. When grafted into the injured spinal cord, OEG can promote the regrowth of axons beyond areas of injury and into distal spinal tissue (see [Section 17.9](#)). The underlying mechanisms are still unclear, but it is possible that OEG demonstrate substantial migration in the host CNS and prevent axons from recognizing the nonpermissive adult CNS environment by ensheathing them, as they normally do for growing olfactory axons. In addition, OEG may support axonal growth by eliciting only a moderate astrocytic reaction.

Stem cells

The application of stem cells for spinal cord repair has to date been limited by technical and ethical concerns and, sometimes, political restrictions. However, different types of stem cells, such as embryonic or adult neural stem cells^{59,60} or stem cell-like bone marrow stromal cells,^{47,61} were shown to elicit neuroprotective and/or regenerative responses. Induced pluripotent stem cells (iPSCs) have also been trialed.⁶² The mechanisms underlying the trophic effects of stem cell transplants are outlined in Chapter 2 **Stem cells**. The secretion of factors that support the repair of existing blood vessels and/or formation of new blood vessels is considered an important determinant in stem cell–based nervous system repair.⁶³ It may be necessary to differentiate stem cells before/during cell transplantation, as the injury environment does not normally favor differentiation into neurons or oligodendrocytes. Differentiation of stem cells prior to implantation is accomplished by a variety of factors (see [Section 17.4.2](#)), although the most efficient combination has yet to be determined experimentally. In vitro differentiation of stem cells or glial restricted precursors to astrocytes via pretreatment with either BMP4 or CNTF results in populations of cells with different properties, which consequently exert different effects on tissue repair when injected into animals with experimental SCI. Astrocytes derived by BMP4 reduce scarring at the lesion site and support axonal regeneration, whereas astrocytes derived by CNTF treatment promote scarring, reduce axonal regeneration, and even induce extreme—often painful—sensitivity to normal stimuli (termed allodynia).^{64,65} It seems clear that not all stem cell or progenitor-derived cells are

beneficial to tissue repair and functional recovery. It has been reported that even intravenously injected stem cells do not need to reach the injured spinal cord to have a beneficial effect. The administered cells are thought to act as decoys to reduce host immune attack on the injured spinal tissue, thus reducing secondary injury effects.⁶⁶ In all instances, it is essential to ensure that stem cells do not continue to multiply after transplantation, which could potentially lead to the formation of tumor-like structures.

Genetically modified cells

Gene therapy holds great potential for the treatment of neurotrauma and many types of neurodegenerative CNS disease. Delivery of therapeutic molecules by gene therapy may support the arrest or delay of secondary neurodegeneration and stimulate the regenerative capacity of CNS neurons. This involves using different cell types including Schwann cells, fibroblasts, OEG, and neural progenitors.

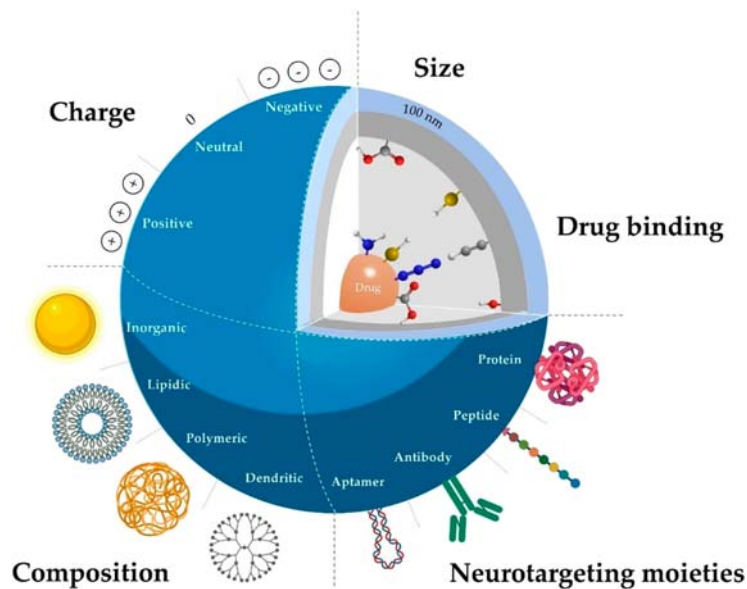
Gene therapy allows selective delivery of neurotrophic factors to specific CNS locations. For example, OEG genetically modified to secrete neurotrophins such as NT-3, BDNF, and GDNF have stimulated long distance growth in certain spinal cord tracts.⁶⁷ In addition, neurotrophin-secreting OEG improve spinal tissue sparing, which may result in improved behavioral outcomes.³⁸ Genetic engineering of cells can involve the use of different viral vector systems and these require hurdles to be overcome for clinical applications. The transfection of donor cells enables better monitoring of the cells within the spinal cord or CNS when using reporter genes. It is also possible to use gene therapy to drive astrocyte-to-neuron conversion in the injured spinal cord using a viral vector to overexpress the transcription factor NeuroD1.⁶⁸

17.4.4 Nanomedicine to treat SCI

Because surgery of the spinal cord often involves substantial risk, relatively **minimal invasive** approaches such as systemic or intrathecal injection are preferred. Nanoparticles (NPs) represent one injectable option and have a high permeability while containing hydrophobic drugs that can be released in a time-dependent manner. Regardless of classification—inorganic, lipidic, polymeric, and dendritic—NPs are able to bind/interact to drugs via functional groups. As portrayed in [Fig. 17.6](#), NPs have tuneable properties such as surface charge (positive, negative, or neutral) and can be functionalized with different targeting ligands, such as aptamers, antibodies, peptides, and proteins.⁶⁹

One example of this is the suspension of methylprednisolone-loaded PLGA NPs in agarose that is administered locally after a spinal cord hemisection.⁷⁰ The delivery of loaded NPs in agarose resulted in improved locomotion, reduced scarring, and decreased inflammation, relative to systemic injection, or local delivery of agarose loaded with blank NPs.

Using NPs targeted at macrophages is another approach to reduce the secondary degenerative event and address neuroinflammation, which is one of the most relevant mechanisms involved in the progression of secondary damage. Polymeric NPs can be used to selectively target macrophages/monocytes due to their specific endocytic/phagocytic activity, because it is known that these cells are recruited to the primary injury zone. Injecting NPs to alter macrophage polarization can reduce the levels of scarring after SCI.⁷¹ NPs offer great potential to target other tissues of the CNS such as the brain (see [Section 17.4](#)).

**FIGURE 17.6**

Main features of the nanoparticles influencing delivery efficiency. From Spencer et al., 2020. *Breaking barriers: bioinspired strategies for targeted neuronal delivery to the central nervous system. Pharmaceutics* 12 (2).

17.4.5 Matrices and scaffolds

An implanted matrix/substrate must allow regenerating axons to penetrate, grow through, and then exit the implant so that the axons can reenter and reestablish connections with appropriate target regions. After SCI, both descending and ascending axonal tracts are disrupted, each of these tracts having specific locations in spinal cord white matter. Since various axonal populations have different growth requirements, a future graft may consist of composite bridging materials, each tailored to a particular axonal population. Whether matrices or scaffolds are effective due to regenerative properties, or as a result of other factors such as neuroprotection, is a matter still under investigation.⁷²

Many biomaterials, either natural (e.g., collagen, alginate, agarose, chitosan, fibrin, fibronectin, Matrigel) or synthetic (e.g., methyl cellulose, nitrocellulose, poly(ethylene glycol) (PEG), nanofibers), encourage some degree of tissue preservation and perhaps fiber tract repair after SCI.³⁵ The implantation of such scaffolds into the injured spinal cord often results in a significantly different injury response to that commonly observed with a traumatic lesion alone. Cystic cavity formation can be completely absent when scaffolds are implanted, and the density of astroglia scarring is often reduced. However, some materials such as collagen Type 1 (in the absence of accompanying donor cells or functional molecules) may induce a detrimental encapsulating response from fibroblast-like cells that interferes with implant-host integration. When such scarring can be reduced or avoided by the use of other materials, scaffolds engineered with oriented frameworks or pores are capable of supporting axonal ingrowth in a highly directional and ordered manner.³⁵

Relevant peptide sequences

For many scaffolds and matrices, there is often a need to direct and target cell/material interactions more specifically than full protein inclusion or adsorption. The conjugation of bioactive peptides with a material is an effective way to influence cell/material interactions, although this has largely been demonstrated only in *in vitro* environments. Many peptides reproduce various bioactive domains of laminin and include YIGSR, IKVAV, RGDS, and RNIAEIIKDI (Arg-Asn-Ile-Ala-Glu-Ile-Ile-Lys-Asp-Ile).⁹ The peptide sequence YIGSR is often preferred as a peptide ligand for *in vitro* applications and is an alternative to RGD for cell adhesion (see Chapter 5 **Extracellular matrix as a bioscaffold for tissue engineering**).

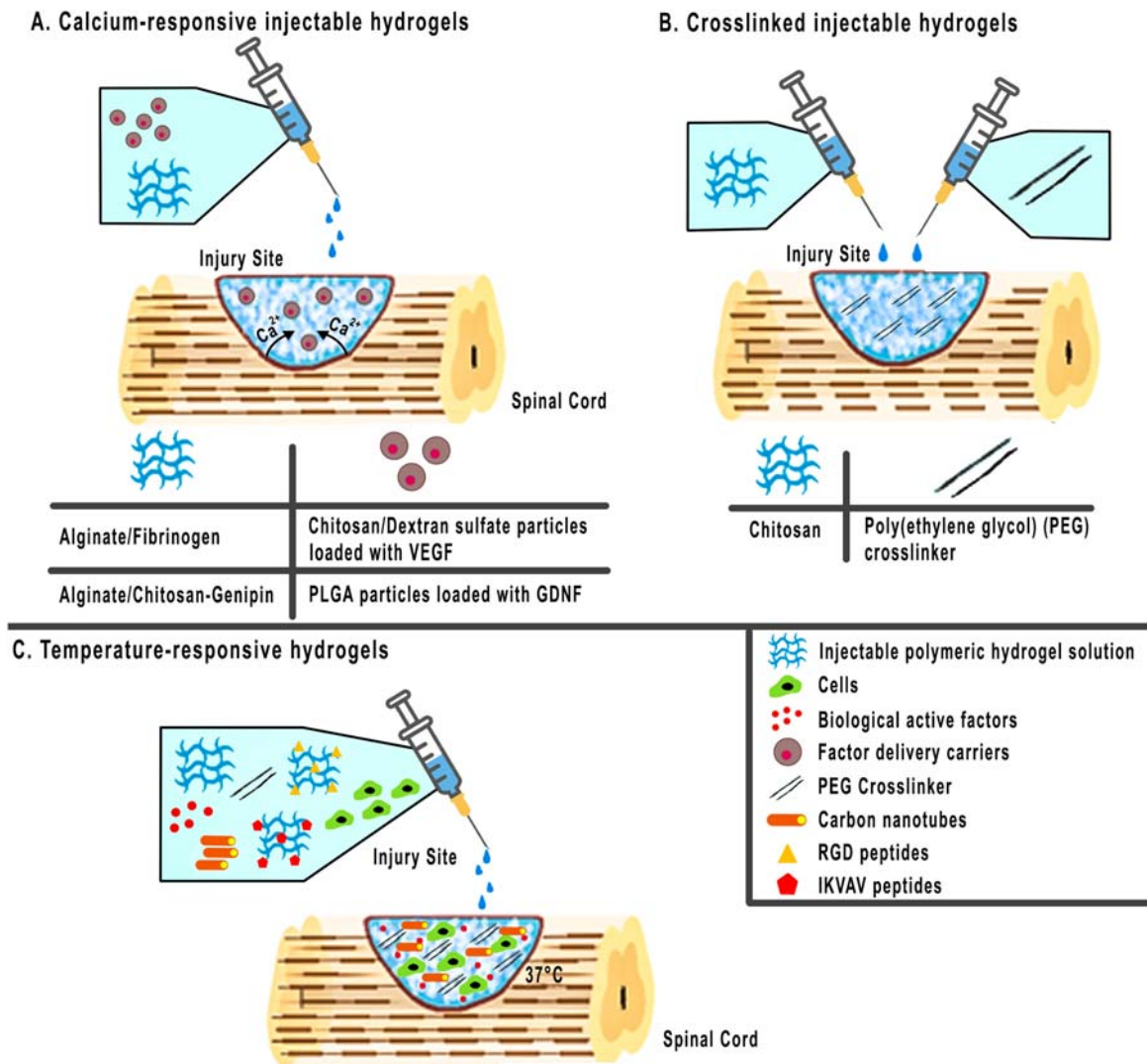
The IKVAV sequence promotes cell adhesion, neurite outgrowth, experimental metastasis, collagenase IV activity, angiogenesis, plasminogen activator activation, cell growth, tumor growth, and differentiation of NPCs.⁹ Although the aforementioned active peptide sequences are effective at promoting neuron adhesion and axonal growth alone, the use of both YIGSR and IKVAV has a synergistic effect on neurite outgrowth. The extended peptides CDPGYIGSR and/or CQAASIKVAV further increase potency⁷³ over the shortened versions but are more expensive to synthesize.

In addition to the conjugation of bioactive peptides with a material, another mechanism of presenting these bioactive sequences is the use of peptide-based materials, such as self-assembling peptides (SAPs). Here, the peptides themselves form the backbone of the material, leading to the high-density presentation of the desired peptide epitope without the need for any additional conjugation steps. This has been demonstrated, for example, by the fabrication of hydrogels containing one or more fluorenylmethoxycarbonyl-(Fmoc-) SAPs, including IKVAV- and RGD-based SAPs, that support neural cell growth *in vitro* and *in vivo*.^{44,74}

Matrices

The number of *in situ* gelling matrices to treat SCI is ever increasing. There are strong arguments for combining cell therapies with injectable matrices to protect cells from membrane shearing and death, similar to issues found in 3D bioprinting. *In situ* gelling hydrogels can be classed as chemically or physically cross-linked, with the latter having a broader range of materials. The types of gelation and reported hydrogels combined with cells and/or biologically active factors used to improve SC regeneration are shown in Fig. 17.7.

In situ gelling matrices are important to maintain a minimally invasive approach to treating SCI. Hyaluronan-Methyl cellulose (HAMC) is being developed as a flexible injectable technology for importing both drugs and cells into the lesioned CNS. Additionally, exploiting the properties of charged SAPs within these gelling matrices offers a platform that can encapsulate neurons and other cells for SCI. Aggregation of the SAP solution after mixing the peptide solution with cell suspensions has been used in numerous SCI studies that demonstrated reduced cavitation and some regeneration.^{75,76} Fibrin is also commonly used as a matrix that can be modified with peptide sequences or used to deliver neurotrophic factors with a heparin binding site.^{23,77} Fibrin also resulted in the greatest number of neurons traced with retrograde labeling in full transection studies, compared to collagen Type I, methyl cellulose, and Matrigel.⁵⁰ Matrigel has also been used in many CNS tissue engineering investigations, particularly with Schwann cell or OEG transplantation into the spinal cord (see Section 17.9). In many of the above-mentioned matrices injected into the spinal cord, reduced scarring is a beneficial outcome.

**FIGURE 17.7**

In situ injectable hydrogels. (a–c) Types of gelation. From Morgado et al., 2019. *In situ injectable hydrogels for spinal cord regeneration: advances from the last 10 years. Biomed Phys Eng Exp* 6 (1).

Scaffolds

Scaffolds made from many different materials have been implanted into the spinal cord.³⁵ Porous scaffolds can be a valuable platform for cell delivery and a tool to elicit neuronal differentiation and functional integration of the delivered cells in the damaged spinal cord.⁷⁸ Alternatively, decellularized tissue has been investigated; however, this is currently limited in scope. Oriented scaffolds, particularly hydrogel scaffolds, have demonstrated their ability to stimulate axonal growth. Similar to scaffolds used for PNI, controlled uniaxial freezing can generate channels within a polymer solution or hydrogels by using the forces associated with crystal growth. The resulting oriented scaffolds demonstrate neurite and cell penetration into the channels.¹²

17.4.6 Nerve guides in the CNS

The use of cell transplantation with nerve guides in the fully transected spinal cord was pioneered by the Bunge group (see Section 17.9) with poly(acrylonitrile-co-vinyl chloride) (P(AN-VC)) nerve guides. Collagen or pHEMA nerve guides can also be used to promote regeneration in the spinal cord.⁵⁰ Regeneration into P(AN-VC) nerve guides occurs with both Matrigel and Schwann cells present in the lumen. As is the case with PN repair, the inclusion of a matrix into a hydrogel nerve guide increases the number of axons.⁵⁰

17.4.7 Summary

SCI initiates a plethora of destructive events. Some of the cells and molecules present at the site of injury may contribute to protection and repair, but the ultimate outcome is loss of nervous tissue and loss of motor, sensory, and autonomic function. Unlike the PNS and the use of PN autografts and nerve bridges, there is currently no reproducible or widely accepted clinical therapy for SCI. One of the main obstacles for regenerating axons in the injured spinal cord is the presence of a spinal injury scar. Repair is also compromised by host immune and inflammatory responses and associated secondary injury cascades that exacerbate any primary injury effects. Therapies that reduce these adverse events will have beneficial effects on spinal cord repair. Cell transplantation combined with supportive matrices and scaffolds or neurotrophic factors has been the predominant focus of tissue engineering strategies. The emphasis has been on neuroprotection, to elicit nervous enhanced tissue sparing, and reestablishment of continuity across an injury site, to reinstate axonal circuits involved in function. Future objectives include increasing the number of damaged ascending and descending axons that regenerate, improving the remyelination of these regenerating axons, the guiding of regenerating axons back to their appropriate target regions in the brain or spinal cord. The development of appropriate animal models for SCI has been a focus of research, with recent endeavors translating to the field of microfluidics that take advantage of small-scale in vitro systems that aim to mimic the disease model. Furthermore, the development of new techniques involving gene therapy and accurate cell dispensing prior to transplantation provides additional approaches to treat this injury. Also critical is where, when, and for how long to apply particular factors.

17.5 CNS: brain

17.5.1 Trauma and stroke

Traumatic lesions to the brain or blockage of blood supply to the brain (stroke) prevent oxygen and nutrient flow. In both clinical conditions, neuronal death occurs almost immediately, and urgent intervention prevents further deterioration. Reconstruction of gray and white matter defects in the brain is especially difficult after large lesions (e.g., stroke or closed head injury) or in the chronic, degenerative situation. In addition to neuronal death, there is demyelination, retraction, and/or aberrant sprouting of injured axons, glial/fibro-adhesive scarring, and there may be progressive tissue cavitation. Under these circumstances, there is a need for cell replacement and some form of neural tissue engineering to develop scaffolds that facilitate reconstruction and restore continuity across a traumatized region.

While some studies have investigated the deployment of biomaterials, usually hydrogels, to act as a bio-bridge from the adult stem cell niche to the injury site,⁸⁹ cell transplantation clinically has demonstrated the most promise.^{90,91} In this regard, biomaterials that have the capacity to provide a safe sanctuary **microenvironment**,^{92,93} while also attenuating inflammation have improved the efficacy of cell transplantation.⁹⁴ For instance, scaffolds have recently been used as biobridges, in combination

with human stem cell transplantation, within a stroke cavity generated in a rat model.⁹⁵ In this instance, SAP hydrogels were used due to their ability to present IKVAV, an epitope prevalent in laminin that is the major extracellular protein in the brain, in high density. The scaffolds supported the human stem cell graft integration, which was associated with continually improved motor function over the 9-month duration of the study, with coordinated paw movement recovering to a level not statistically separable to intact controls. When human stem cells were delivered in combination with the SAP scaffold, the grafts were larger; there was superior neuronal differentiation and subsequent integration, as demonstrated via electrophysiology data.⁹⁵

17.5.2 Neurodegenerative diseases

Neurodegenerative diseases are often associated with substantial neuronal loss, the pattern of loss varying dependent on the type of disease process. Supply of appropriate neurotrophic factors via grafted donor cells or from slow-release polymers may protect compromised host neurons, but alternative cell replacement strategies may also be required.

Some of these neurodegenerative disorders, such as Huntington's disease (HD) and spinocerebellar ataxia, are inheritable and associated with single gene mutations. Other motor neuron disorders and diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) have more complex etiology, with only a percentage directly associated with dysfunction of a specific gene. It is likely that many of these degenerative conditions are affected by environmental factors as well as some form of genetic susceptibility. AD and PD in particular are diseases associated with the elderly and are becoming increasingly common as life expectancy increases and the population ages.

Early diagnosis and prevention of further deterioration would be the optimal ways of treating these neurodegenerative disorders. However, it is often the case that when symptoms become apparent there has already been substantial neuronal death. Engraftment of encapsulated cells that secrete the appropriate growth factor(s) has been trialed in experimental models of PD, AD, HD, amyotrophic lateral sclerosis (ALS) (loss of cortical motor neurons and spinal cord alpha-motor neurons), and chronic pain.⁹⁶

Other tissue engineering strategies involve attempts to replace the lost neurons with new cells to be incorporated into host circuitries at either physiological or ectopic locations. PD is a multisystem disease, a major feature being the catastrophic loss of neurons in the midbrain that secrete dopamine. In numerous clinical trials, hundreds of PD patients have received intracerebral grafts of dopamine-rich fetal human brain tissue, many of these recipients experiencing some alleviation of their symptoms. Potentially alternative sources of tissue include xenografts and stem cells or NPCs modified to secrete dopamine. For example, human pluripotent stem cells have been ectopically transplanted into the brain of mice using a tissue-specific hydrogel. Importantly, this hydrogel was programmed to support these progenitor cells in two different ways. Firstly, they were rationally designed to be biocompatible with dopaminergic precursor cells and in fact biased the differentiation of grafted cells to see a 51% increase in a specific population of neurons lost in PD and critical for motor function. Secondly, controlled and sustained presentation of GDNF within the graft core was achieved, inducing a 2.7-fold increase in dopaminergic neurons and enhanced plasticity. Taken together, this resulted in significant restoration of motor function in parkinsonian animals over a 6-month duration.⁹⁷ Such approaches, perhaps combined with neuroprotective gene therapy, are potentially also of use in the treatment of motor neuron diseases and HD, where some recent studies have utilized biomaterials to improve the efficacy of viral vector gene therapy.⁹⁸

17.5.3 Drug delivery to the brain

To reach specific areas of the brain, a non- or minimally invasive delivery of the therapy is key. The use of NPs as delivery systems for difficult-to-access parts of the body is one exciting area of research, while the imaging of tumors using NPs is another active area of research.⁹⁹ For many drug delivery systems to the CNS, the BBB is a key aspect to consider, confounded by the fact that many systemically injected NPs are rapidly removed from circulation. However, there are instances when the permeability of vasculature is significantly altered through injury or disease. One example is the period following injury or stroke, where there is a temporary increase in BBB permeability.¹⁰⁰ A cell surface receptor for self-recognition, CD47, has been recombinantly produced and attached onto NPs to significantly increase circulation times.¹⁰¹ Despite the selective barrier function of the BBB, a number of papers have demonstrated that NPs can cross from the bloodstream into the brain, although there is evidence that serum protein binding to NPs (protein corona) can be problematic.¹⁰²

Alternatively, NPs can be delivered to the CNS via intrathecal delivery¹⁰³ within an in situ gelling hydrogel to control their release. Intranasal delivery is also noninvasive route for delivering NPs to the brain and drug delivery in general.¹⁰⁴ More recently, studies have exploited the diffusion kinetics of drugs from biomaterials to achieve localized delivery of multiple drugs simultaneously, while importantly avoiding systemic delivery and the many problems associated with off-target effects. For instance, biomaterials have been deployed on the surface of the cortex to deliver both cyclosporin and erythropoietin simultaneously, with the drugs being shown to be localized and bioavailable for at least 32 days. This resulted in increased plasticity in the striatum and stimulation of endogenous NPCs in a relatively minimal invasive manner.¹⁰⁵ Such approaches, including localized and controlled gene therapy, are critical to the future of brain repair research to replicate embryogenesis and/or promote adult neurogenesis.

Brain targeted therapies

In the brain, successful delivery of drugs requires that they bypass the various barriers that separate the bloodstream from the brain **parenchyma**.⁶⁹ As described above, the BBB presents the major obstacle in systemic brain therapies due to the strong tight junction between endothelial cells, as portrayed in [Fig. 17.8](#). This endothelial layer is regulated primarily by astrocytes and pericytes. To overcome the BBB, drugs may be directly delivered to the extracellular space with a catheter. This administration route takes advantage of the spaces between cells (interstitial pathway) to attain a homogeneous perfusion throughout the brain. This approach has been successfully employed with different types of delivery nanosystems, such as polymeric NPs. NPs may also be delivered to the brain via intraarterial injections, which take advantage of the major blood supplier to the brain, the carotid artery.

Overcoming the physical barriers that separate circulation between the body and the brain can further be paired with neural-targeted delivery. The successful delivery of drugs to neurons depends on the presence of exclusive receptors called neurospecific receptors. Other considerations are the competitive nature of other molecules, or ligands, that bind to the same receptor. Ligand–receptor interaction strength greatly influences the delivery rate, which is further influenced by the optimal ligand density, ligand mobility, and correct exposure of the targeting ligands. Choosing the correct ligand–receptor and tailoring the factors that affect drug delivery is crucial for developing effective therapies.

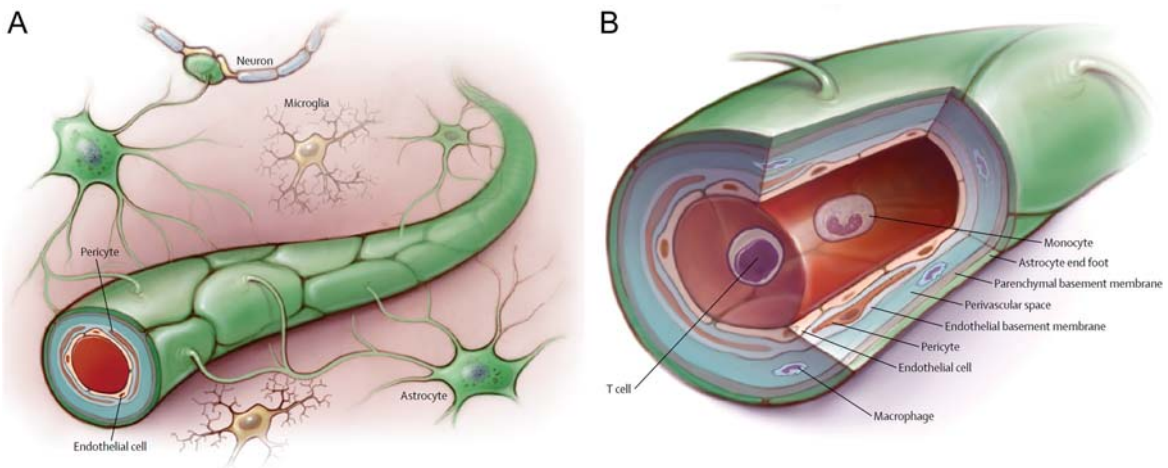


FIGURE 17.8

Schematic of the neurovascular unit. The blood–brain barrier separates CNS tissue from circulating blood and limits the effective intravenous delivery of many pharmaceuticals. From Pollak, T.A., et al., 2018. *The blood brain barrier in psychosis. Lancet Psychiatr* 5 (1), 79–92.

17.5.4 Bioprinting for the brain

An early approach to incorporate neural cells within biomaterial-based inks to create bioprinted scaffolds demonstrated the possibility of 3D coculture of rat embryo neurons and astrocytes incorporated in collagen.⁸¹ More recently, mouse primary cortical neurons were incorporated in a gellan gum bioink modified with RGD, a peptide sequence known to improve cell adhesion. As a result, 3D brain-like structures were successfully developed via extrusion-based bioprinting.¹⁰⁶

Recent studies using a bioink that combined alginate, chitosan, and agarose have demonstrated successful in situ expansion and sequential differentiation of human iPSCs.¹⁰⁷ iPSCs derived from somatic cells are reprogrammed into a pluripotent state and can bypass issues associated with immune rejection and ethical concerns when compared to stem cells. Human iPSCs were successfully differentiated using the compound bioink described above, resulting in brain-like tissue with functional migrating neurons and neuroglia. This study presents perhaps a cornerstone that contributes to the potential of bioprinting in fabricating structures that aim to mimic functional neural networks in the brain.

17.6 CNS: optic nerve

Damage to fiber tracts interconnecting different CNS regions causes disruption of neural circuits and often leads to the death of associated neurons. As already mentioned for SCI, therapeutic strategies are therefore needed to ensure the viability of affected neurons, promote the regrowth of damaged axons across lesion sites, maintain growth within CNS tissue distal to the injury, and finally ensure that regrowing axons reinnervate appropriate target areas and reconnect with relevant target neurons.

The visual system is a useful model¹⁰⁸ in which to study regenerative success or failure after nerve fiber damage in the mature CNS since

- (i) Retinal ganglion cells (RGCs), the neurons that project axons out of the retina through the optic nerve and via the optic chiasm and optic tracts to central targets in the brain, can be directly targeted via intraocular injections.
- (ii) There is detailed knowledge of intraretinal and visual brain circuitry.
- (iii) The optic nerve is a discrete, centrally derived tract that is surgically accessible and can be manipulated without affecting other fiber tracts and structures.
- (iv) The extent of RGC survival and axonal regrowth can readily be quantified.

Normally, mature mammalian RGCs do not regrow axons beyond an optic nerve injury and, if the axotomy occurs within the orbit, about 90% of RGCs die within 2 weeks. This lack of regrowth is due to factors both intrinsic and extrinsic to the adult RGC.¹⁰⁹ However, some adult RGCs will survive axotomy and regrow their axons when appropriately stimulated and/or when provided with an appropriate local environment.

17.6.1 Regenerative therapies

After optic nerve crush, adult RGC survival can be enhanced by intraocular injections of recombinant neurotrophic factors such as BDNF, NT-4/5, CNTF, or GDNF or by injections of factors that block intracellular signaling pathways associated with cell death. However, these effects are transient, and viability is in most cases enhanced for only a few weeks. Use of viral vectors, particularly adeno-associated viral (AAV) vectors, encoding appropriate growth promoting genes to transduce and genetically modify RGCs provides more long-term protection.¹¹⁰

Some regrowth of RGC axons across an optic nerve crush can be elicited after implantation of peripheral nerve fragments or MSCs into the vitreous, injuring the lens, or recruiting and activating monocytes/macrophages within the eye. Some regeneration is seen after blockade of cell-intrinsic molecules that suppress axonal growth, for example, after inactivation of Rho, a GTPase that inhibits growth cone motility (see Chapter 4 **Cellular signaling**), or by modification of relevant transcription factors and/or intracellular signaling pathways.^{108,109} Overall, without combinatorial approaches that use several complementary therapies, adult RGC axons fail to regrow more than just a few millimeters past the injury site.

An approach that permits substantial long-distance regrowth of injured adult RGC axons has been to autograft PN segments from the same animal onto the cut optic nerve. Under these conditions, regenerating axons reinnervate target areas in the brain and some limited function restored. However, there is a functional cost involved in removing the nerves for transplantation. Alternatives are therefore being sought including nanotechnology approaches, matrices containing cultured OEG and chimeric PN grafts containing genetically modified Schwann cells.^{111–113} Various types of hydrogel matrices, some containing peptide and aminosugar sequences, have also been used in attempts to promote the regeneration of RGC axons after more central optic tract injuries in the brain.¹¹⁴

The optic nerve injury model provides an optimal system in which to examine combinatorial effects as pharmacological agents, recombinant growth factors or viral vectors can be injected into the eye to directly influence RGCs, while tissue and genetic engineering can be used to modify the environment within which

RGC axons regenerate. These interactions can be assessed at a single location using different combinations of factors, or at multiple locations—for example, testing the effect of factors applied to the cell body and supplying cells/appropriate factors near the growing tips of the axons.¹¹⁶ Combined pharmacotherapeutic and vector-mediated strategies have been reported to drive more extensive regrowth of a proportion of RGC axons after optic nerve crush injuries, sometimes beyond the optic chiasm and at least as far as the optic tracts.¹⁰⁸ Note that, as in all CNS repair studies, great care must always be taken to distinguish between spared axons and axons that are truly regenerating after an injury.¹¹⁷

17.7 CNS: retina

The retina is a thin, layered neural structure consisting of diverse populations of RGCs, bipolar and amacrine cells, horizontal cells, photoreceptors (cones and rods), Müller glia, and retinal pigment epithelial (RPE) cells, the latter attached to a thin membrane called the Bruch's membrane (Fig. 17.9a). Bruch's membrane is partially synthesized by the RPE cells and contains laminin, collagen type IV, fibronectin, and heparin sulfate proteoglycans. RPE cells maintain the health and functionality of the photoreceptors. When the RPE and photoreceptors are detached from Bruch's membrane, photoreceptor cell death (and visual loss) ensues.

17.7.1 Diseases of the retina

Diabetic retinopathy, retinitis pigmentosa, and macular degeneration are three major diseases of the eye, while glaucoma—often associated with raised intraocular pressure—results in damage to the optic cup and retina. Age-related macular degeneration (ARMD) is the leading cause of blindness in the United States for people aged above 55 years. Current effective treatments for retinal disorders are limited, and a range of potential tissue engineering strategies exist.^{118,119} These strategies often center around cell transplantation, from different sources and are sometimes accompanied by a scaffold or a matrix.

17.7.2 Cell transplantation

Intraocular cell transplantation is one therapeutic strategy for the treatment of degenerative diseases that primarily affect photoreceptors. Different transplantation methods (retinal sheets, fragments, dissociated mixed or purified cell suspensions) have been trialed, and different sources of cells have been used, including neural precursors, embryonic and postnatal retinal precursor cells, photoreceptors, neural stem cell lines, and bone marrow stem cells.^{120–122} Fig. 17.9b shows how RGC-like cells are introduced to the eye/retina in an attempt to replace host neurons and/or repair optic nerve damage.

One tissue engineering strategy for ARMD is the transplantation of RPE cells to the subretinal space. A major challenge is that when RPEs are injected into the subretinal space as a suspension, they are unable to form ordered monolayers and eventually die. Cell transplantation substrates are therefore used as carriers to deliver cells, such as RPEs, iris pigment epithelium cells, and retinal progenitor cells to the retina (Fig. 17.9c). The seeded substrates are typically inserted subretinally through a slit in the sclera and choroid. Injected cell suspensions are exposed to high shear forces as they pass through the injecting needle, while therapies involving supporting substrates avoid such stresses. The use of cells on scaffolds for transplantation to the retina thus has advantages over injection of cells as spheres or single cell suspensions, where large numbers of cells do not survive the transplantation procedure. Many different transplantation substrates have been investigated, including natural and synthetic biomaterials, biohybrids,

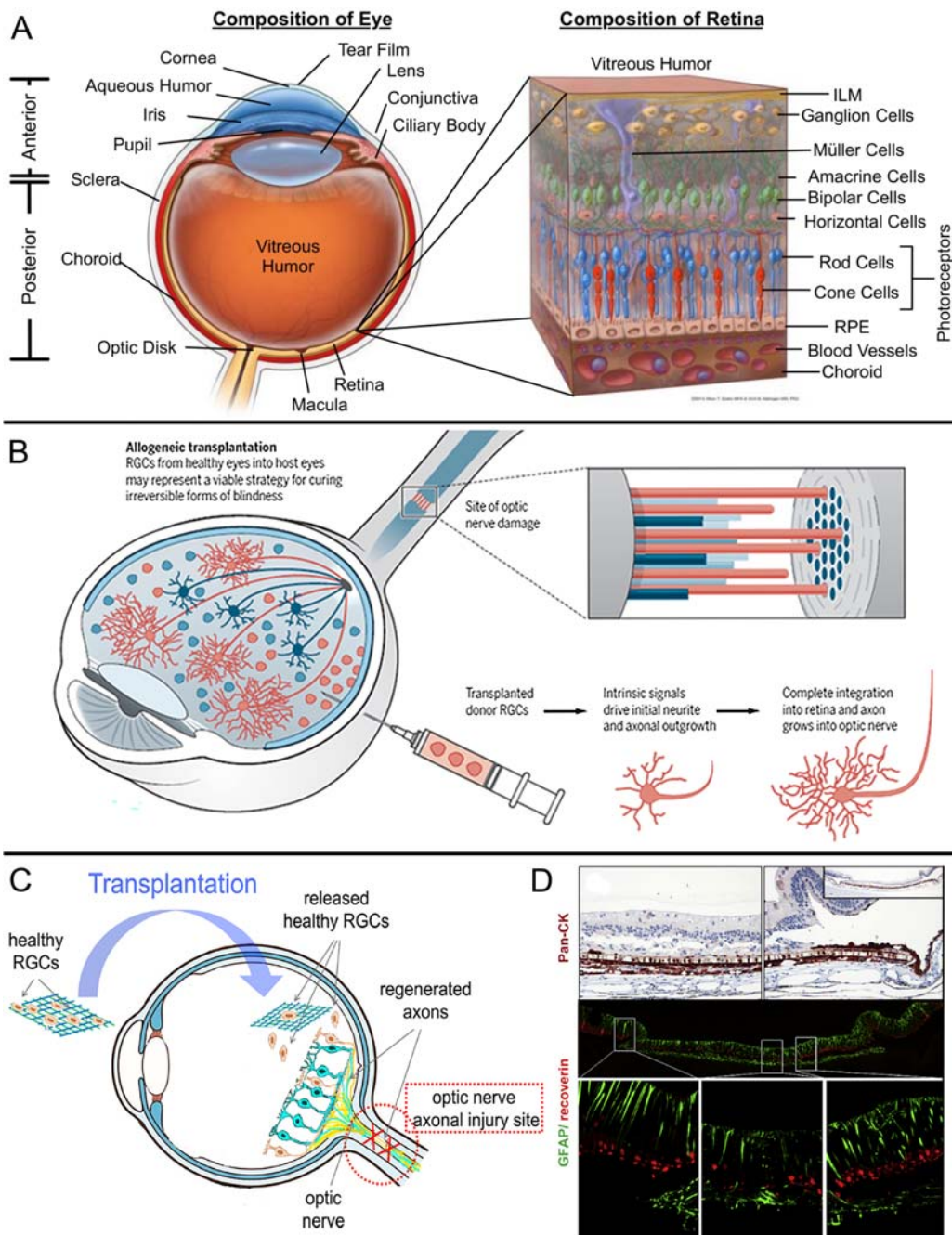


FIGURE 17.9

(a) Expanded view of the eye showing the layers of the retina. (b) Transplantation of injected cells into the eye and intended repair outcomes. (c) Transplantation of RPE-seeded scaffold for cell delivery. (d) RPE-seeded electrospun scaffolds transplanted subretinally in rabbits. (a) From Parsons, D.E., et al., 2021. Peptidomimetics therapeutics for retinal disease. *Biomolecules* 11 (3), 339. (b) From Behtaj, S., et al., 2020. Retinal tissue bioengineering, materials and methods for the treatment of glaucoma. *Tissue Eng Regenrat Med* 17 (3), 253–269. (c and d) From Liu, Z., et al., 2014. Enhancement of retinal pigment epithelial culture characteristics and subretinal space tolerance of scaffolds with 200 nm fiber topography. *Biomaterials* 35 (9), 2837–2850.

and various methods of microfabrication. Fig. 17.9d shows an example involving the implantation of electrospun sheets.¹¹⁵ In this figure, RPE cells are brown, rod photoreceptors are red, and Müller glial cells are green. Substrates for subretinal implantation need to be a relatively thin thickness (the Bruch's membrane has an average of 5 μm) but require a certain amount of mechanical integrity to withstand the placement and implantation procedures. Research is ongoing to find an ideal and effective cell transplantation scaffold, including via 3D bioprinting,¹²³ for optimal delivery of stem cells or precursor cells.^{119,124}

17.8 Future perspective

The injury response and healing mechanisms of the PNS are different to those of the CNS. The main challenge for PNS regeneration remains the reconnection of severed nerves that are separated from each other above a critical gap length. Fibers, cell transplants, and scaffolds continue to be investigated and some significant gap lengths are now being bridged with electrospun sheets. Oriented scaffolds with morphological similarities to structures within which the bands of Büngner form distal to the PN injury are often researched. Ultimately, the advances in scaffold fabrication processes such as 3D printing may provide the high-resolution scaffold needed for "fascicle-to-fascicle" guided regeneration. Any tissue engineering solution for the PN must improve on the widely used autograft surgery.

In situ gelling materials for the brain and spinal cord maintain their importance over the next decade for the delivery of therapeutics. Preformed or injectable hydrogels are often considered simple, while in fact they are highly complex, performing multiple roles required for treating the nervous system. These gels will perform increasing functions, including the survival/differentiation of transplanted cells and the delivery of NPs for targeted therapeutic delivery. Automated systems such as in bioprinting will continue to improve and allow more sophisticated matrix/bioink delivery.

The types of cell available for transplantation are also likely to be greater in the future, including perhaps activation of the patient's own endogenous neural precursor cells. The production of a successful regenerative bridging construct in the CNS requires knowledge of the dynamic temporal changes that occur after an injury, an understanding of the complex molecular and cellular environment in each injured area, and strategies to overcome this inhibitory environment. Combining cell transplantation in the contused spinal cord with other repair-promoting interventions such as pharmacotherapy and gene therapy allows substantially more "tools" for neurological recovery. Surgery can also be performed on transgenic animals (particularly mice), providing insights on the role of certain genes in regeneration with tissue engineered constructs. A major challenge will be to develop tissue engineering protocols that are suitable for use in chronic as well as acute injury situations. Further understanding of the effect of scaffold implantation will continue to provide tissue engineers with new concepts for bridging CNS defects and may one day provide an "off-the-shelf" solution to spinal cord repair.

The extensive use and positive safety data for mRNA delivery for COVID-19 vaccines will undoubtedly be directed toward therapeutics for the nervous system. Neural tissue engineering can play a central role in multitherapeutic delivery for combinations of nucleic acid delivery, cell transplantation, and drug release. The optic nerve is an excellent CNS model to test a combination of therapies, and this could become a model organ to investigate combinatorial therapies. In addition to the optic nerve, the retina is a tissue with surgical accessibility via the vitreous cavity. For this tissue, strategies in delivering polymeric sheets for cell transplantation to the retina will have important ramifications in the delivery of

tissue engineering strategies to the eye. Improved prosthetic devices that improve activation of the central visual pathways will also advance over the next decade.

More diverse neurological injuries and diseases are likely to be tackled with tissue engineering strategies. Approaches for the treatment of brain trauma and stroke are likely to involve combinations of therapies and may be distinct from therapies developed to treat degenerative diseases. Diseases such as multiple sclerosis and stroke may also respond to NP delivered drugs in clinical settings, particularly if systemic or intrathecal delivery can be a route for treating CNS injuries and disorders. The next decade should also see the translation of tissue engineering therapies to the clinic for treatment of injuries beyond the PNS. It is even possible that the long-time “holy grail” of assisting people suffering from SCI will be achieved in the next decade using some aspect of these bioengineering principles.

Summary

- Current approaches to successfully aid PN regeneration after PNI remain limited to a critical gap length, currently 30 mm for humans.
- Matrices and scaffolds have been explored to mimic an autologous nerve transplant, the typically used approach to promote nerve repair. The resulting nerve guides may be loaded with cells, functionalized with neurotrophic factors and/or combined with gene therapies.
- The inflammatory response that occurs after SCI may be managed using cell loaded or neurotrophic-functionalized scaffolds. Further, the use of inhibitors has also been employed to aid the inflammatory response. Hydrogels have been proposed as a minimally invasive strategy for their delivery.
- Nanomedicine via the use of NPs has been proposed as an approach to bypass the naturally occurring barriers to the CNS.
- Biofabrication techniques have developed throughout the recent years to produce matrices and scaffolds that better mimic the mechanical and physiological properties of native tissue for nerve regeneration.
- Regeneration of the optic nerve requires combinatorial approaches for sufficient RGC axonal regrowth. The optic nerve has been proposed as an ideal CNS model to test such combination of therapies.

Classical experiment

Schwann cell and OEG transplantation in P(AN-VC) nerve guides

The use of Schwann cell transplants within nerve guides for spinal cord repair was pioneered in the Bunge laboratory at The Miami Project to Cure Paralysis during the 1990s. Autologous Schwann cells can be isolated and cultured from peripheral nerves of the SCI patient. The potential of Schwann cell—filled nerve guides for spinal cord repair was demonstrated by Xu and coworkers,⁵³ who transplanted a poly(acrylonitrile-co vinyl chloride) P(AN-

VC) tubular scaffold seeded with Schwann cells and Matrigel into the completely transected adult rat thoracic spinal cord. Several weeks after transplantation, myelinated and unmyelinated axons were present within the Schwann cell cable that connected the opposing spinal cord stumps (Fig. 17.10a–c). These and other experiments demonstrated the potential of using Schwann cells for spinal cord repair. A drawback is that responding axons grew across the Schwann cell graft but did not exit the graft to grow into the spinal cord distal to the implant, which

Classical experiment—continued

prevented the formation of new synaptic connections and, thus, new axonal circuits that are needed to control motor function. Increasing the level of neurotrophins just caudal to the SC transplant supports axonal growth beyond the implant. Fig. 17.10 visually depicts axonal growth across a Schwann cell-filled P(AN-VC) tube and neurotrophin facilitated exit of the axons in hemisection models. When there is Matrigel only (i.e., no Schwann cells) in the P(AN-VC) tube, there is limited growth of axons into the transplant. While Schwann cell inclusion with the Matrigel demonstrated axonal growth, the axons were only successfully guided out of the nerve guide via the delivery of BDNF and/or NT-3 into the caudal spinal cord a few millimeters from the implant.⁵⁴

In the olfactory system, there is continued penetration of newly formed axons into the CNS, even in adulthood. OEGs enfold and guide these elongating olfactory axons so that the axons are unaffected by any growth inhibitory cues in the CNS environment. This unique feature of OEG

was thought to be beneficial for eliciting axonal growth across and beyond an injury site. While OEG will ensheath axons, they do not myelinate them as is the case with Schwann cells. In pivotal experiments at The Miami Project to Cure Paralysis, OEG and Schwann cells were combined. A P(AN-VC) tube was filled with Schwann cells mixed in Matrigel and implanted into the adult thoracic spinal cord. OEGs were then injected into the rostral and caudal stumps. The results showed that the additional injections of OEG at either end of the Schwann cell channels allowed greater numbers of axons to exit the Schwann cell bridging grafts.^{53,55} Descending axons such as those of the raphe system (located in the brainstem) grew in areas devoid of many Schwann cells but were seen in areas containing fibroblasts and OEG. Sensory axons also regenerated for long distances—up to 18 mm. Such an experiment demonstrates the utility of multiple treatments for a successful spinal cord repair outcome.

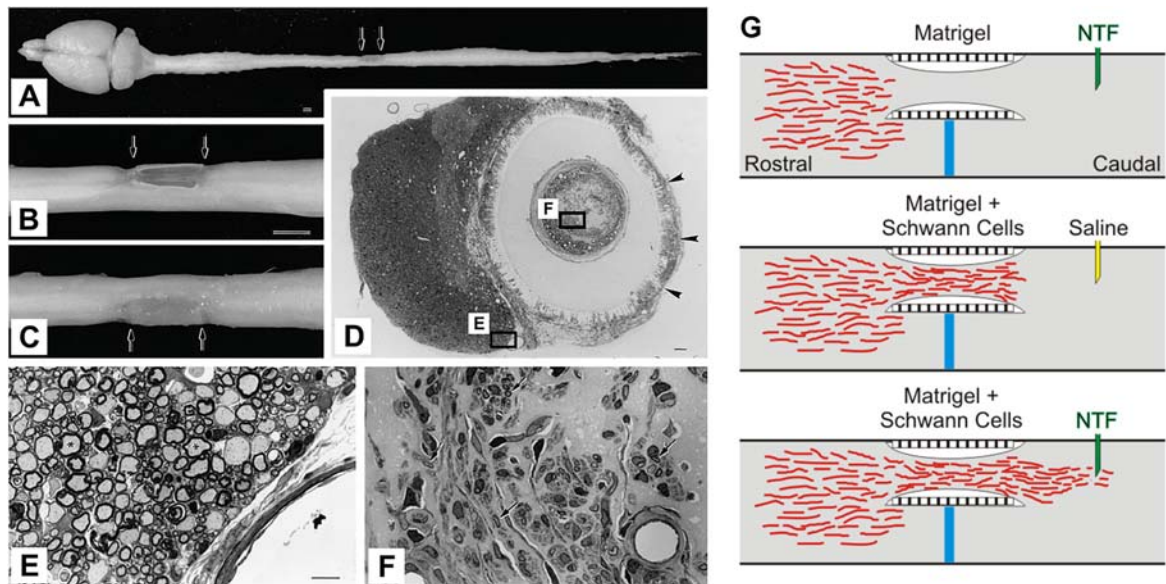


FIGURE 17.10

Schwann cell/Matrigel transplants in P(AN-VC) guides in a hemisected spinal cord. (a and b) Photographs of brain and spinal cord (a), and closer magnifications of the hemi-section and implant (b and c). (d) Transverse section depicting the tissue bridge and the intact contralateral spinal cord ventral and dorsal horn. (e and f) Myelinated axons (e) and smaller caliber axons (f) in the tissue cable. (g) Illustration of the effect of P(AN-VC) nerve guides implanted within hemisections. (a–f) From Xu, X.M., et al., 1999. Regrowth of axons into the distal spinal cord through a Schwann-cell-seeded mini-channel implanted into hemisected adult rat spinal cord. *Eur J Neurosci* 11 (5), 1723–1740.

State-of-the-art experiment

Bioprinting for the nervous system

The highly complex architecture of the nervous system requires cells to be accurately placed in a 3D environment when attempting to form structures that mimic neural networks. The automated and controlled placement of cells can be achieved by processing cell-laden biomaterials into a predesigned 3D scaffold with a technique called bioprinting, which has emerged as an area of focus for tissue engineering of the nervous system. Throughout this chapter, several examples in which bioprinting were applied to a particular tissue of the nervous system were introduced. The chronological development of such findings is summed up in Fig. 17.11.

In the first approach to bioprint nerve cells, primary hamster embryonic motor neurons were delivered onto a collagen gel-based biopaper (Fig. 17.11a).⁷⁹ The placement of these cells into a circular pattern using a modified Hewlett Packard desktop inkjet printer conferred the potential for high throughput and cost-effective way to position neural cells. The disadvantages of this technique include low ink cell density, discontinuous flow, and high working temperatures for thermal inkjet printer processing. Inkjet bioprinting may alternatively rely on piezoelectric forces, a setup which has been used to bioprint adult rat retinal ganglion cells and glia. Inkjet-based bioprinting is still restricted by height, which underlines the need to develop new techniques that would allow the fabrication of cell-laden constructs in a 3D environment.⁸⁰

Neural cells were later incorporated into a hydrogel biomaterial-based ink (or simply, bioink) to develop 3D scaffolds in a layer-by-layer approach (Fig. 17.11b).⁸¹ The 3D coculture of rat embryo neurons and astrocytes incorporated in collagen was important to demonstrate the possibility of coculture, which is relevant for the eventual establishment of neural networks *in vitro* given the range of cell types that make up neural tissue, and also the important interactions between neural and nonneural cells that naturally coexist in the body. This co-culture approach was later applied to RGD-modified gellan gum which incorporated primary cortical neurons into a brain-like structure printer layer-by-layer (Fig. 17.11b). The first approach to improve bioinks by combining different materials applied to the nervous system involved

the extrusion of cell-laden fibrinogen into a thrombin solution, which led to the successful bioprinting of rat Schwann cells (Fig. 17.11c).³²

In a first approach to coculture neural cells, human iPSC—derived neural progenitors were incorporated within an alginate and methyl cellulose scaffold matrix and successfully led to neural cell maintenance and proliferation (Fig. 17.11d).⁸² In a separate study, cell-laden biomaterials were successfully tailored to the dimensions of the rodent spinal cord, demonstrating the ability to reengineer neural cell responses using digital light processing, a 3D printing technique also called microscale continuous projection printing (Fig. 17.11e).⁸³

Development of other bioprinting techniques such as in gel/support bath printing has successfully been translated and optimized to neural applications throughout the years. Resorting to a gelatin support bath was initially explored in a cell-free approach to print cortical-like structures. More recently, this approach was paired with human neuroblastoma cells incorporated in a sodium alginate bioink, paving the way toward applying this technique to study neural interactions in bioprinted brain-like structures.⁸⁴

Time-dependent structural transformation of bioprinted scaffolds adds a fourth dimension to bioprinting.⁸⁵ 4D approaches which incorporate external stimuli postprinting have recently been applied to advance the complexity of nerve guides. Nerve guides incorporating human MSCs were fabricated onto a reprogrammable multiresponsive graphene structure and have shown promising neuroregeneration ability after implanted in rats.⁸⁶ 4D-based bioprinting has further been proposed as a powerful tool for a more in-depth study of neuronal mechanical processes that occur in the cortical folds of the brain.⁸⁷

Moving forward, bioprinted scaffolds will combine novel bioinks and drug-releasing polymers with 3D fine tailored microfluidic architecture to carefully engineer neural cell fate (Fig. 17.11f).⁸⁸ Taken together, the examples portrayed along the timeline shown in Fig. 17.11 illustrate the ever-developing field of bioprinting of nerve cell laden scaffolds toward multi strategic approaches of bioprinting for applications in the nervous system.

State-of-the-art experiment—continued

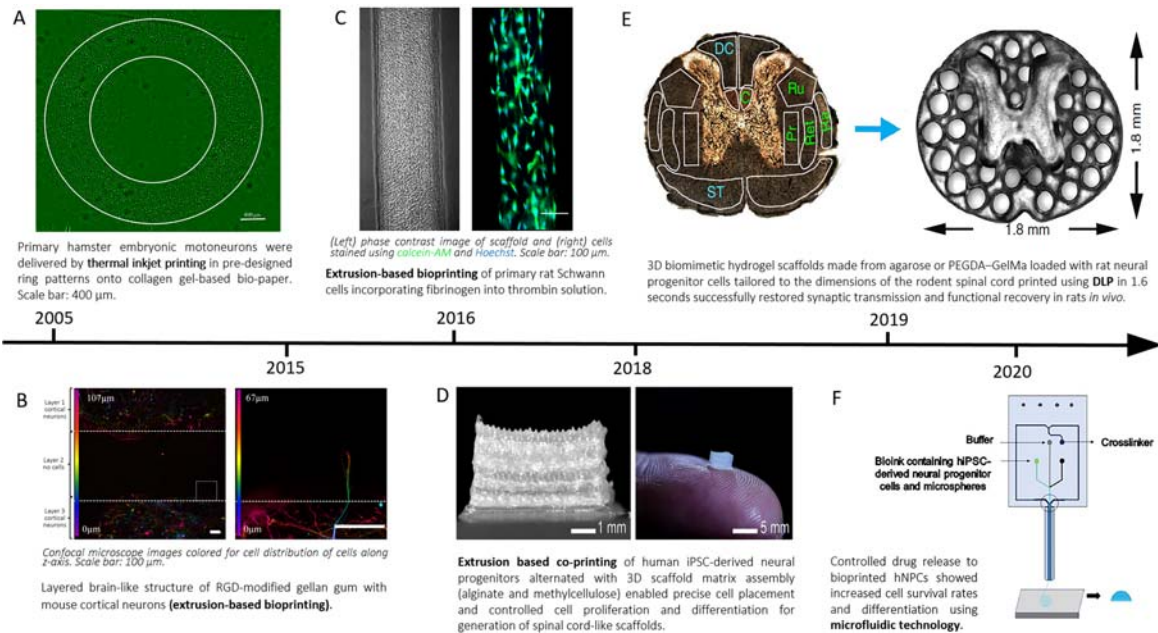


FIGURE 17.11

Selected examples of milestones through advancements in bioprinting applied to the nervous system. (a) From Xu, T., et al., 2005. Inkjet printing of viable mammalian cells. *Biomaterials* 26 (1), 93–99. (b) From Lozano, R., et al., 2015. 3D printing of layered brain-like structures using peptide modified gellan gum substrates. *Biomaterials* 67, 264–273. (c) From England, S., et al., 2017. Bioprinted fibrin-factor XIII-hyaluronate hydrogel scaffolds with encapsulated Schwann cells and their *in vitro* characterization for use in nerve regeneration. *Bioprinting* 5, 1–9. (d) From Joung, D., et al., 2018. 3D printed stem-cell derived neural progenitors generate spinal cord scaffolds. *Adv Funct Mater* 28 (39). (e) From Koffler, J., et al., 2019. Biomimetic 3D-printed scaffolds for spinal cord injury repair. *Nat Med* 25 (2), 263–269. (f) From Sharma, R., et al., 2020. 3D bioprinting pluripotent stem cell derived neural tissues using a novel fibrin bioink containing drug releasing microspheres. *Front Bioeng Biotechnol* 8, 57.

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17.10 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
1. What is a main difference between sensory and motor neurons?
 2. What is the role of Bands of Büngner?
 3. Autograft approach is the “gold standard” to treat PNI when the gap between the damaged nerves is too large for the nerves to be reattached. List one disadvantage of the autograft approach.
 4. Define critical gap length.
 5. What is the approximate critical gap length for humans?
 6. Once a PN nerve guide is sutured into position, an important protein naturally builds up due to the microenvironment response to the implant. What protein is this bridge made of?
 7. Name four important neurotrophic factors in nerve regeneration.
 8. Compared to PN, there is one main obstacle for regenerating axons in the CNS following injury to the spinal cord. Please identify this process.
 9. What is one approach that could be employed to address the spinal injury scar that forms after SCI?

10. Animals are used to model different degrees of SCI. List one model used for SCI.
 11. What is one main advantage of using NPs to treat SCI?
 12. NPs, matrices, and scaffolds may be functionalized with peptide sequences that aim to promote and direct neuronal growth. List one.
 13. Name one advantage and one disadvantage of using cats as animal models for SCI.
 14. Cell transplants have been proposed as a therapeutic approach to treat SCI. Name one cell type.
 15. What is the difference between 3D and 4D biofabrication?
 16. Name one 3D printing technique applied to the nervous system.
 17. A proposed alternative to bypass the blood–brain barrier (BBB) is to deliver drugs intranasally. Name two advantages of the intranasal delivery route.
 18. Describe one way in which the visual system is a useful model to study regenerative success or failure after nerve fiber damage in the mature CNS.
 19. One main goal after optic nerve crush is to enhance the survival of retinal ganglion cells (RGCs). Neurotrophic factors have been proposed, but what is a more long-lasting alternative?
 20. Age-related macular degeneration (ARMD) has been tackled by transplanting retinal pigment epithelium (RPE) cells to the subretinal space.
 - 20.1. What is one main challenge when suspended RPEs are injected into the subretinal space?
 - 20.2. What is a proposed alternative to overcome this challenge?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:
1. Current approaches to treat PNI include the use of nerve guides. Some commercially available nerve conduits degrade shortly after being implanted in the body (as little as 3 months), whereas others are designed to not degrade. What are the advantages and disadvantages of degradable (both short and long term) versus nondegradable nerve guides?
 2. Describe how you would design an experiment that incorporates a multifaceted approach to tackle PNI.
 3. How would a therapeutic approach differ when addressing the peripheral versus the central nervous system? (PNS vs. CNS)
 4. Even within the CNS, the physical properties of the nervous tissue vary greatly in physical/mechanical properties. How do you expect to tailor the mechanical properties of scaffolds or matrices that are to be implanted in the brain versus the spinal cord, for example?
 5. Injury to the spinal cord caused by trauma creates a local primary response after impact, which then triggers a cascade of secondary inflammatory responses. Outline the existing tissue engineering approaches that aim to tackle the later stages of SCI.
 6. What are the main considerations when designing a therapeutic strategy for degenerative diseases versus trauma-related injuries to the nervous system?
 7. Wallerian degeneration is a degenerative process that occurs after injury to an axon. Sketch a figure that compares a healthy axon to one undergoing Wallerian degeneration.

8. If you reflect on the concepts presented in this chapter, how would you argue the importance of having a team of multidisciplinary researchers and clinicians to develop new therapeutic approaches for nerve regeneration?
9. In your opinion, what is still a major challenge in tissue engineering of the nervous system?
10. What do you expect will be a major advance in the field of tissue engineering of the nervous system in the next 5–10 years?

Challenge-based learning

Designing nerve guides for pain management

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Pain is an essential human sensation to alert us to adverse conditions, but patients suffering from chronic pain have a much reduced quality of life, and in their situation, pain does not have an important signaling function. Pain management is a very active field of research and a current concept is that the immune system and peripheral sensory nervous system communicate by molecular signaling. When the nerve is severed in the case of amputees, painful neuromas form, however nerve guides appear to reduce the risk of this occurring.

Motivation and stakeholders

Pain sensitivity can be regulated/dysregulated by the immune system. Macrophages are able to promote and resolve pain sensitization and also T-cells play a dual role in pain sensitization. In addition to this, molecular therapeutic tools are available, such as small molecules, therapeutic antibodies, and siRNA, that can modulate the physiology of the immune system. It is therefore interesting to look into expanding on the opportunities to treat pain in amputees by using a tissue engineered implant. Future therapies should be based on molecular insight into the interplay between the immune- and nervous systems. Such therapies should be designed with the input from stakeholders such as intensive care unit doctors, nurses, physical therapists, mechanical engineers, immune engineers, and neural cell biologists.

Problem definition

Amputees suffer from painful neuromas that are regenerating nerves that are unable to find targets and bundle together.

Challenge

To develop a nerve guide that is specifically engineered to control nerve regeneration and prevent neuromas, potentially in conjunction with an external prosthetic.

Learning framework

Reading the Neural Tissue Engineering chapters and related literature will help you to understand the following:

1. Division, anatomy, and physiology of the peripheral nerve system
2. The sensory nerve system as part of the peripheral nerve system, and how the body feels pain.
3. The current state of peripheral nerve guides.
4. Conditions that exacerbate pain and how pain can evolve from acute to chronic.
5. The immune system as a pain mediator and the role of macrophages and T-cells.
6. Mechanisms of action of macrophages and T-cells to modulate pain.

For a more detailed look into your project, make a mind map summarizing the following:

7. Mechanism of action of current pain management drugs such as steroids and antiinflammatory drugs.
8. Specific dimensions of your nerve guide, for the different animal models.
9. The molecular signals that combine to stimulate or reduce nerve regeneration.

Challenge-based learning—continued

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

17.11 Glossary

Allogenic means taken from different individuals of the same species.

Alpha Motor Neurons are nerve cells, the cell bodies of which are found in the CNS, with axons that extend toward the periphery to innervate muscle and cause muscle contraction.

Apoptosis is a form of programmed cell death that occurs in multicellular organisms.

Autonomic nervous system controls the function of organs and glands; is separated into sympathetic and parasympathetic components.

Axon is a long, slender projection of a nerve cell that typically conducts electrical impulses known as action potentials away from the nerve cell body.

Axotomy refers to severing of an axon.

Biofabrication is the production of complex biologic products from living cells, matrices, biomaterials, and molecules.

Bioinks are carrier materials used to produce an engineered tissue using 3D printing.

Bioprinting is the utilization of 3D printing techniques to combine cells, growth factors, and/or biomaterials to fabricate biomedical parts, often with the aim of imitating natural tissue characteristics.

Blood–brain barrier is a highly selective semipermeable border of endothelial cells that prevents solutes in the circulating blood from nonselectively crossing into the extracellular fluid of the central nervous system where neurons reside.

Central nervous system (CNS) is the part of the nervous system consisting primarily of the brain and spinal cord.

Central pattern generator refers to neural circuits that produce rhythmic motor behavior without rhythmic input in activities such as walking or breathing.

Chemotactic is the movement of an organism or entity in response to a chemical stimulus toward the source of the stimulus.

Contusion injury is a tissue injury where the capillaries are damaged by trauma, causing localized bleeding that extravasates into the surrounding interstitial tissues.

Critical gap length is defined as a nerve gap over which no recovery will occur without the use of nerve grafting or bridging.

Critical-sized defects are defined as those that will not heal spontaneously within a patient's lifetime.

Distal refers to the (injured) part of the tissue away from the neuron cell body.

Donor site morbidity refers to complications and functional restrictions that the patient has to undergo because of harvesting tissue from a healthy donor site.

Fascicle-to-fascicle repair is a surgical technique to precisely match fascicles to recover nerve function.

Full transection is a complete interruption of white matter tracts, segmental gray matter, and associated nerve roots in the spinal cord.

Gamma Motor Neurons are nerve cells, the cell bodies of which are also found in the CNS, that take part in the process of muscle contraction by monitoring muscle length and stretch.

Glia are nonneuronal cells of various types that perform a wide range of support functions in the peripheral nervous system (PNS) and normal CNS.

Hemisection spinal cord injury (SCI) model is a tissue injury model characterized by damage to one half of the spinal cord.

In situ means the location where it occurs under normal circumstances.

Intrathecal space is the fluid-filled area located between the innermost layer of covering (the pia mater) of the spinal cord and the middle layer of covering (the arachnoid mater).

Microenvironment is the micrometer range environments of cells.

Minimal invasive surgery is a surgical technique that limits the size of incisions needed to lessen wound healing time, associated pain, and risk of infection.

Myelin is the insulation around axons that speeds up the conduction of nerve impulses.

Necrosis is unprogrammed cell death due to cellular damage or infiltration by pathogens, as opposed to orderly programmed cell death via apoptosis.

Nerve guides are conduits between the severed proximal and distal nerve stumps to provide structural and trophic support.

Neuropathological condition is a disease of the nervous system.

Neurotrophic factors are a family of biomolecules that support the growth, survival, and differentiation of both developing and mature neurons. Neurotrophic factors are sometimes called neurotrophins.

Oligodendrocytes are a type of neuroglia whose main function is to provide support and insulation to axons in the central nervous system.

Parenchyma is the functional part of the tissue. In the nervous tissue, the parenchyma excludes, for example, fluid-filled spaces, blood vessels, or meningeal tissue (tissue that protect the brain and spinal cord).

Peripheral and Cranial Nerves contain nerve fibers (axons) that interconnect the central nervous system (CNS) to the periphery.

Peripheral nerve injuries (PNIs) occur when nerves of the peripheral nervous system are damaged due to physical or environmental factors or disease (e.g., accidents, falls, trauma, or diabetes).

Peripheral nervous system (PNS) consists of the nerves and ganglia outside the brain and spinal cord that reach organs and tissues like the heart, intestines, bones, and muscles.

Proximal is the (injured) part of the tissue closer to the neuron cell body.

Retrograde degeneration is a pattern of neuron destruction following axonal injury that spreads backwards along the axon, toward, and then into the nerve cell body.

Schwann cells are the myelin producing cells in the peripheral nervous system.

Sensory Neurons are nerve cells that carry sensory information from the body extremity to the CNS.

Spinal cord injury is damage to the spinal cord.

Spinal injury scar develops after spinal cord injury and consists of multiple cells and extracellular debris, with axonal growth inhibitory molecules to form a physical and chemical barrier for regenerating axons.

Wallerian degeneration is an active process of degeneration that results when a nerve fiber is cut or crushed and the part of the axon distal to the injury (i.e., farther from the neuron's cell body) degenerates.

Xenogeneic a tissue or organ that is derived from, originating in, or being a member of another species.

© Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Principles of cardiovascular tissue engineering

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18.1 Learning objectives

After reading this chapter you will be able to:

- Understand the pathophysiology of myocardial infarction (heart attack) leading to heart failure, and the inability of the current standard treatments to induce regeneration of the damaged heart tissue.
- Define the strategies of tissue engineering and how they can be implemented to target processes leading to myocardial regeneration, including cardiomyogenesis, modulation of extracellular matrix remodeling, and angiogenesis.
- Explain the two major components of cardiovascular tissue engineering: cells and biomaterials.
- Know the diverse applications of biomaterials, e.g., for the delivery of transplantable cells, controlled release of bioactive molecules, and as scaffold for tissue engineering.
- Describe the challenges in bioengineering of vascularized cardiac patches in vitro and the biotechnology tools (scaffolds and perfusion bioreactors, 3D bioprinter) employed to achieve functional engineered tissue.

The intrinsic inability of the heart to effectively repair itself after major ischemic insults, such as myocardial infarction, urgently calls for continuous extensive research for finding the appropriate strategies to induce myocardial regeneration and repair.

Ruvinov et al. (2012)

Myocardial tissue engineering encounters a variety of challenges, which are grouped into three categories associated with cells, materials and vascularization.

Qi-Zhi Chen et al. (2008)

18.2 Introduction

Tissue engineering is of particular interest for the treatment of heart diseases, such as after **myocardial infarction** (MI), or due to congenital heart defects, where large portions of functional tissue are lost with very limited intrinsic regeneration ability. Such loss often leads to significant and progressive deterioration in cardiac contractility and function, eventually resulting in the development of life-

threatening conditions and **heart failure** (HF). The shortage of transplants and donors, the inability of current clinical therapies to restore functional myocardial tissue loss after MI, and the urgent need for functional cardiovascular tissue substitutes are the main factors contributing to the enormous efforts being invested in development of cardiovascular tissue engineering and regeneration strategies.

In this chapter, we present the basic principles of cardiovascular tissue engineering, providing the readers with essential information regarding the advanced and effective strategies for cardiovascular repair and regeneration.

18.3 Heart structure, disease, and regeneration

18.3.1 The myocardium

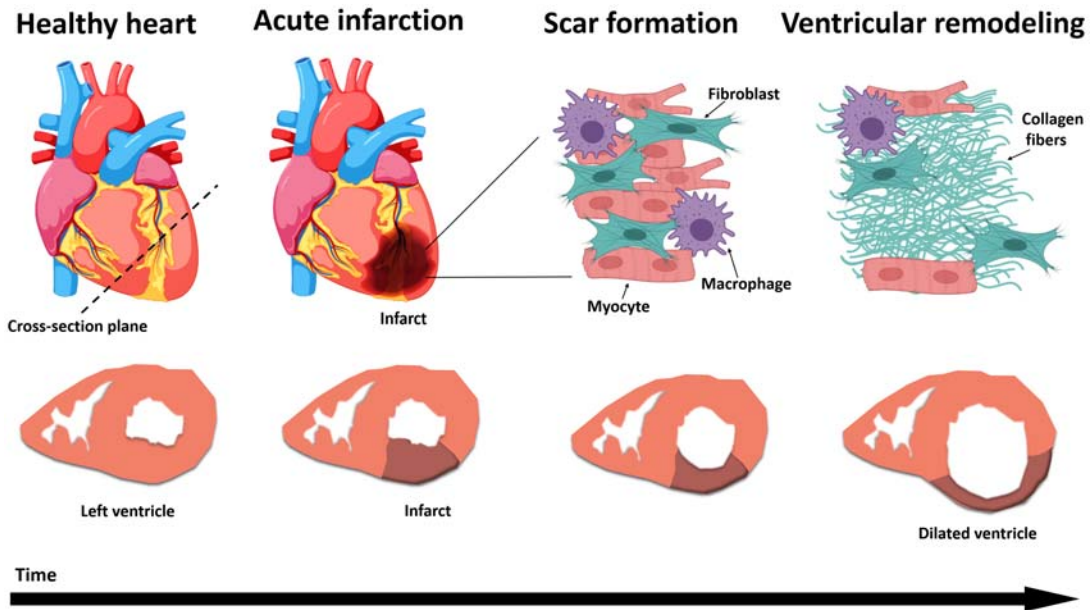
The heart is a vital organ, which is indispensable for maintaining normal homeostasis of the organism. The **myocardium**, i.e., the cardiac muscle tissue, constitutes about 95% of the heart mass and is responsible for its pumping action. The human myocardium consists of 2–3 billion **cardiomyocytes (CMs)** (75% by volume, 30% by number); they are striated muscle cells found only in the heart which can be visually distinguished from the skeletal and **smooth muscle cells (SMCs)**. There are many differences between cardiac and skeletal muscle. Both have striations, but beyond that, the former has special unique features which contribute to their functional effectivity. The heart is **myogenic**, making it self-sufficient, whereas skeletal muscle is controlled by the nervous system.

18.3.2 Myocardial infarction and heart failure

Coronary heart disease (CHD) is now the leading cause of global deaths, affecting millions of people worldwide. MI is the most common manifestation of CHD, accounting for ~50% of all CHD cases in the United States. MI results from temporary or permanent occlusion of the main coronary arteries, causing significant reduction in blood supply to the beating heart muscle (mainly to the left ventricle (LV)).

In a healthy heart, the LV pumps newly oxygenated blood to the rest of the body, and its walls are normally thick with cardiac muscle fibers. When blood supply to the beating muscle is reduced as a result of coronary artery occlusion, the myocardium (cardiac muscle) tissue becomes **ischemic** (is not getting enough blood and oxygen), CMs die from oxygen deprivation, and an infarct develops. Because of its high metabolic rate, the myocardium begins to undergo irreversible injury within 20 min of ischemia. Within hours and days, **extracellular matrix (ECM)** degradation takes place. The infarct is infiltrated by macrophages and collagen-producing myofibroblasts. The infarcted wall of the ventricle becomes thin and rigid. As healthy myocytes die at the border of the scarred area, the infarct expansion continues. Developed pressure overload is initially compensated by **hypertrophy** (growth in muscle size) of the healthy myocardium. Ultimately, however, this compensating mechanism fails, and infarct wall thinning, and ventricle dilatation continues. As a result, the heart is unable to pump effectively, leading to the life-threatening condition of HF (Fig. 18.1).

Five partially overlapping phases can be distinguished in the progression from healthy to infarcted myocardium: (1) CM loss due to an initial ischemic event, (2) acute inflammatory response, (3) ECM remodeling, (4) granulation tissue formation, and (5) scar formation and LV remodeling. The structural remodeling of the heart (i.e., infarct wall thinning, scar formation, and noninfarcted myocardium hypertrophy) leads to LV functional remodeling, a progressive deterioration in heart muscle function that eventually leads to HF.

**FIGURE 18.1**

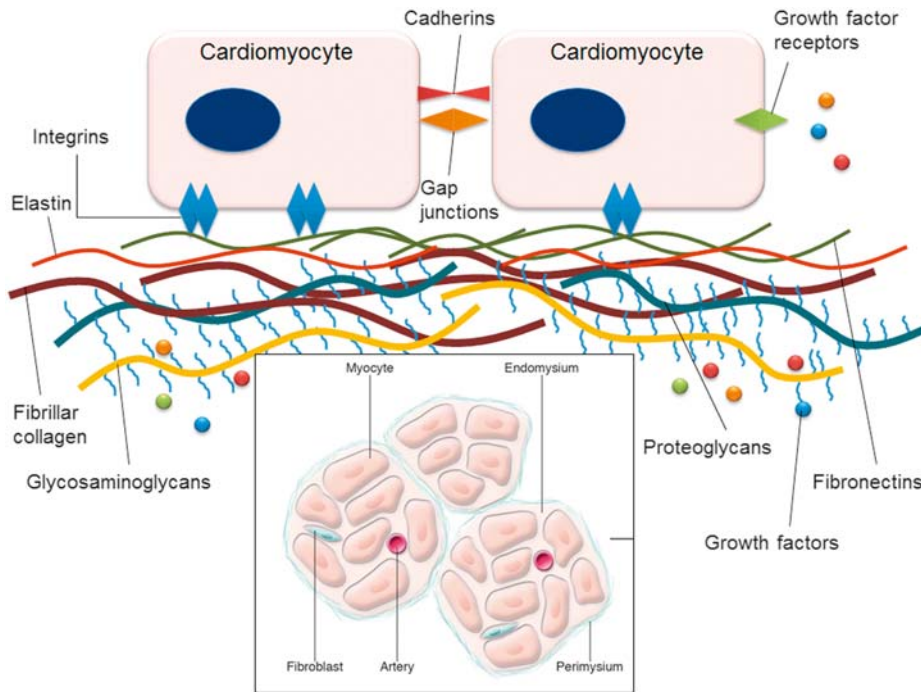
Heart failure: from acute crisis to chronic disease. *Created with BioRender.com.*

Over recent decades, major improvements have been realized in the management of patients with MI. These include in-hospital treatments (e.g., pharmacological lysis, antiplatelet, and antithrombin therapies), interventional therapies, and surgery (e.g., cardiac catheterization, percutaneous coronary intervention, coronary artery bypass surgery, and heart transplantation) as well as drug regimens for prevention and long-term treatment (e.g., aspirin, ACE inhibitors, β -blockers, and statins). The improved management of acute coronary events, however, has led to a significant increase in the number of patients that suffer from chronic conditions, namely HF.

A major disadvantage of these therapies is their inability to replace, at least partially, cardiac muscle after infarction. Thus, there is a need for alternative approaches that enable to overcome the limitations of standard therapies. The ultimate goal of such novel therapies is the induction of myocardial tissue regeneration (therapeutic, endogenous, or combined), in situ or ex vivo.

18.3.3 Cardiac ECM—function and pathological changes after MI

The architecture of the myocardium is very complex. CM orientation and myocardial fiber angles throughout the LV free wall are highly organized and move in a continuous fashion from the **endocardium** (inner layer) to the **epicardium** (outer layer of the heart). The cardiac ECM plays a crucial role in maintaining the unique CM orientations. A network of proteins with highly organized structure and architecture, such as type I and type III collagen, provides structural integrity to adjoining CMs and contributes to overall LV pump function through the coordination of CMs shortening.

**FIGURE 18.2**

A schematic presentation of the myocardial environment and major ECM components. *From Ruvinov et al., Cardiac tissue engineering: principles, materials, and applications. In: Synthesis Lectures on Tissue Engineering; 2012.*

Fig. 18.2 shows a scheme of the myocardial environment. Generally, the major ECM components in the myocardium include fibrillar collagen, fibronectin, elastin, proteoglycans, and glycosaminoglycans (GAGs) (see Chapter 5 **Extracellular Matrix as a Bioscaffold for Tissue Engineering**). The ECM–cell interactions are mediated by integrins on the cell surface, while the cell–cell interaction complex comprises cadherins and gap junction proteins. The ECM also serves as a controlled reservoir of growth factors.

Collagen fibers surrounding individual CMs and collagen struts tethering adjacent CMs comprise the endomysium—the delicate connective tissue surrounding the individual muscular fibers. Groups of CMs are bundled within the perimysium—the connective-tissue sheath that surrounds a muscle, and capillaries and coronary microvessels have free diffusion access to CMs throughout the ECM. The main components of the myocardial ECM and myocardium are listed in Table 18.1.

Significant alterations in the structure and composition of the myocardial ECM occur following MI, which eventually affect the mechanical properties of the LV myocardium. Very early post-MI, the normal ECM within the ischemic region is degraded. As the post-MI period progresses over the next several days, an influx of inflammatory cells into the injured myocardium occurs, which results in further proteolysis of cellular and ECM proteins. In addition, this inflammatory response causes

Table 18.1 Components of the myocardium and myocardial ECM.

Component	Main function
Collagen fibrils (types I and III)	Structural support, maintain shape Force transmission Tensile strength (type I); resilience (type III)
Elastin	Resilience; vessel wall stretch; cardiac wall stretch; and relaxation
Hydrophilic glycosaminoglycans	Diffusion of nutrients, metabolites, growth factors, cytokines, etc.
Proteoglycans	Mechanics, fluid dynamics
Integrins (matrix receptors)	Myocyte-fibroblast/ECM interactions, matrix remodeling
Fibronectin and laminin	Adhesive fibrous proteins
Cells	
Cardiomyocytes	Cardiac muscle cells, responsible for contractile function of the heart
Fibroblasts	Production of fibrillar collagen Differentiate to myofibroblasts after injury
Macrophages	Phagocytosis, inflammatory response
Other cells	Endothelial cells, smooth muscle cells, pericytes, neurons

From Jugdutt. Ventricular remodeling after infarction and the extracellular collagen matrix: when is enough enough. Circulation. 2003; 108(11). Fomovsky et al., Contribution of extracellular matrix to the mechanical properties of the heart. Cell Cardiol. 2010;48(3).

proliferation and differentiation of fibroblasts and other interstitial cells, and the elaboration of bioactive molecules which contribute to a robust synthesis of ECM for the purposes of scar formation. The later phase of post-MI remodeling results in ECM changes within all regions of the LV: the MI region, the viable myocardium within the border zone, and the remote region. This adverse remodeling process has been termed “**infarct expansion**” and occurs in the absence of additional myocyte injury or alterations in LV loading conditions. It has been postulated that an acceleration of ECM degradation occurs within the myocardium surrounding the MI (border zone) facilitating the infarct expansion process in this later phase of post-MI remodeling. This post-MI remodeling process is a clinically significant problem in that it can lead to LV dilation, systolic and diastolic dysfunction, and progression to HF. Indeed, this process, which includes changes in ECM structure and composition, is an independent predictor of morbidity and mortality.

18.3.4 Endogenous myocardial regeneration

Neonatal mammalian hearts, including mice, rats, and even humans, can exhibit the ability to regenerate and functionally recover in several injury models, in a limited time window immediately after birth. However, in the human adult heart after a major MI, the regeneration rates are extremely low. This can explain the lack of significant restoration of cardiac muscle after severe ischemic injury, the formation of fibrotic scar at the infarct, and progressive deterioration in cardiac function, which eventually lead to CHF.

18.3.5 Potential therapeutic targets and strategies to induce myocardial regeneration

In order to compensate for the low and insufficient intrinsic regeneration ability of the adult heart, strategies for therapeutic regeneration aim to induce myocardial regeneration, improve tissue salvage, facilitate self-repair, reverse or attenuate adverse remodeling, and ultimately achieve long-term functional stabilization and improvement in heart function. Five major processes associated with MI are targeted at present by various experimental regeneration strategies (Fig. 18.3):

- *Cardioprotection*—the prevention of progressive CM loss following MI by applying various apoptosis-inhibiting reagents or by inducing prosurvival signaling.
- *Inflammation*—time-adjusted modulation of the post-MI pro/antiinflammatory cytokine/chemokine profile or cellular responses (e.g., granulation tissue formation and macrophage infiltration) to induce effective tissue healing and repair and to avoid negative inflammatory effects (e.g., cell death, fibrosis, etc.).
- *ECM remodeling and cardiac fibrosis*—time-adjusted positive modulation of the fibrotic response (i.e., ECM remodeling and scar formation), utilizing recent knowledge on profibrotic signaling, such as modification of the ratio between metalloproteinases and their inhibitors, which may lead to successful antifibrotic therapy.
- *Angiogenesis*—increasing the blood supply to ischemic regions is an extensively used approach for effective tissue healing. A variety of proteins, genes, or cells have been tested, aimed at inducing the formation of new vasculature at the infarct site.

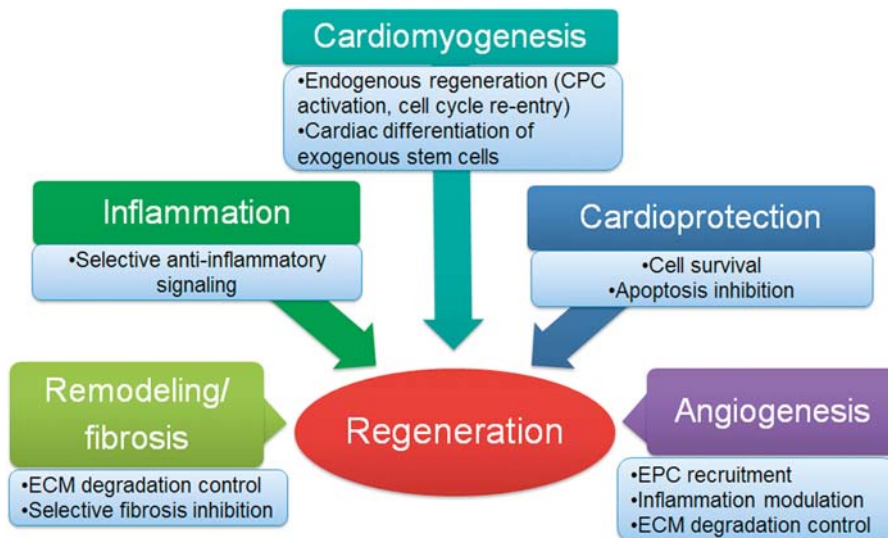


FIGURE 18.3

Therapeutic myocardial regeneration strategies (see text for details). From Ruvinov et al., *Cardiac tissue engineering: principles, materials, and applications*. In: Synthesis Lectures on Tissue Engineering; 2012.

- **Cardiomyogenesis**—myocyte regeneration by activation and/or migration of distinct cell populations with stem- or progenitor-like properties in the adult myocardium which can contribute to de novo myocardium formation after MI. An alternative novel mechanism for endogenous myocyte regeneration is the induction of CM cell cycle reentry by reprogramming of differentiated CMs toward proliferation.

18.4 Cell sources for cardiovascular tissue engineering and regeneration

Cells represent a critical component in the tissue engineering paradigm. While preclinical research utilizes many readily available cell sources (cell lines, animal-derived isolated cells, etc.), translation of the developed strategies into the clinic requires human and preferably autologous cell sources of functional cardiovascular cells.

The major cell types used for cardiovascular tissue engineering and regeneration are as follows (Fig. 18.4):

- **Autologous** (derived from the same individual) endothelial cells (ECs) and SMCs—nonimmunogenic autologous ECs and SMCs isolated from the patients themselves are the first choice for use in blood vessel engineering.

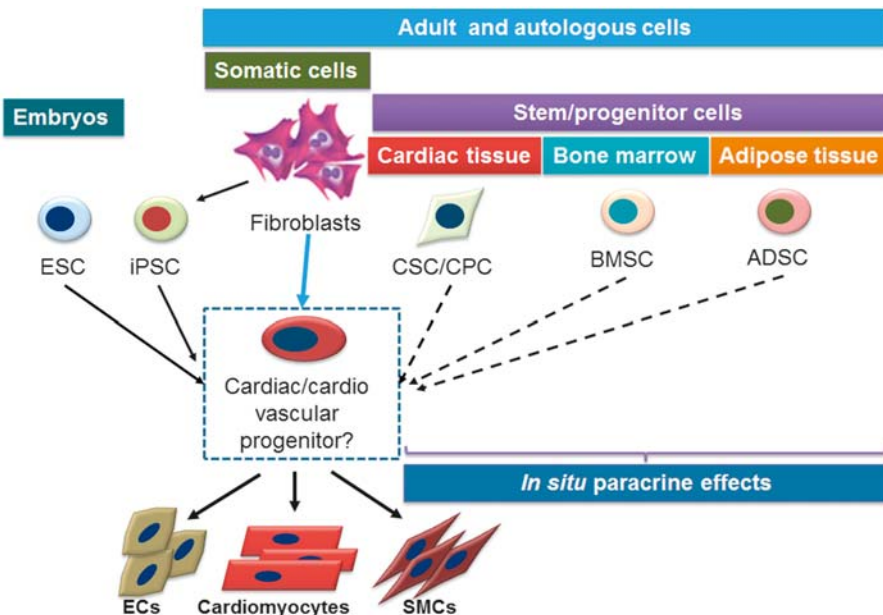


FIGURE 18.4

Clinically relevant cell sources for cardiovascular tissue engineering and regeneration. From Ruvinov et al., *Cardiac tissue engineering: principles, materials, and applications*. In: *Synthesis Lectures on Tissue Engineering*; 2012.

- **Pluripotent** (capable of differentiating into one of many cell types) stem cell—derived CMs—human CMs produced by directed differentiation of pluripotent stem cells (embryonic stem cells or induced pluripotent stem cells (iPSCs) that can be derived from somatic cells (e.g., fibroblasts)). More advanced and future clinical applications of these cells will require refinement of differentiation protocols and/or inclusion of maturation steps.
- Pluripotent stem cell—derived vascular cells—human pluripotent stem cells could also be used for the derivation of vascular cells (ECs and SMCs).
- Mesenchymal stem cells (MSCs)—represent a contemporary alternative in scenarios where human functional cells (e.g., CMs) are unavailable. MSCs can be isolated from bone marrow or adipose tissue.
- Cardiac stem/progenitor cells (CSCs/CPCs)—numerous CSC/CPC pools within the adult heart have been characterized on the basis of stem cell marker expression and cardiomyogenic potential.

18.5 Biomaterials—polymers, scaffolds, and basic design criteria

Biomaterials and scaffolds are critical components in cardiovascular tissue engineering and regeneration strategies, as standalone or in combination with cells and/or bioactive molecules (Fig. 18.5).

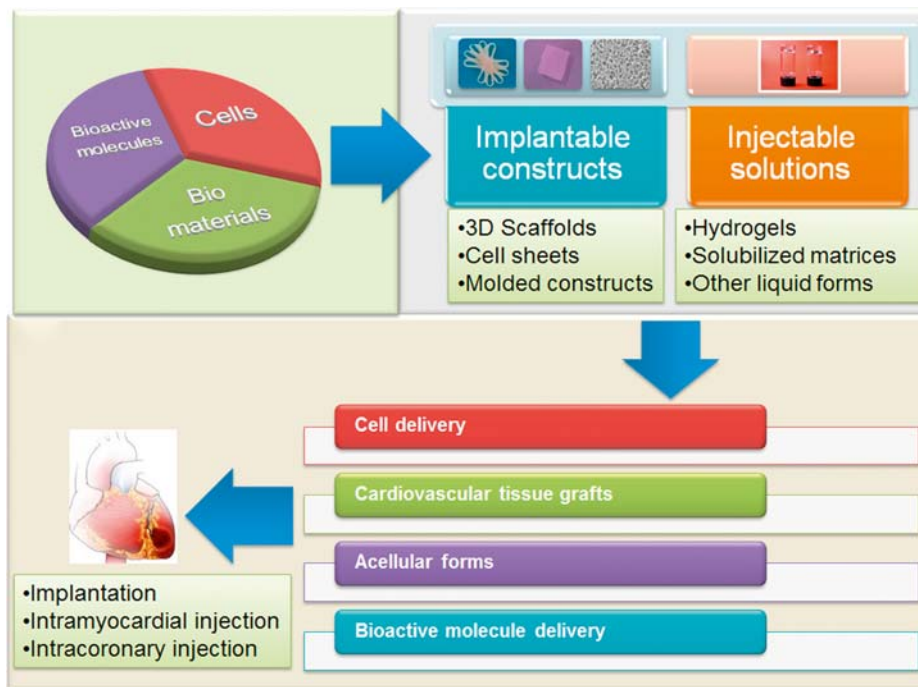


FIGURE 18.5

Paradigms for the use of biomaterials in cardiac tissue engineering and regeneration. From Ruvinov et al., *Cardiac tissue engineering: principles, materials, and applications*. In: Synthesis Lectures on Tissue Engineering; 2012.

They have been used as follows: (1) ECM replacement in acellular strategies; (2) controlled delivery systems for bioactive molecules; (3) vehicles for cell delivery and enhanced retention in the damaged tissue; and (4) supporting and guiding matrix for cell organization into a functional tissue, *in vitro* and *in vivo*.

In general, the biomaterials used in cardiovascular tissue engineering and regeneration strategies should comply with the following basic criteria:

- *Biocompatibility*—The ability of a scaffold to perform as a substrate supporting the appropriate cellular activity, including the facilitation of molecular and mechanical signaling, in order to optimize tissue regeneration, without eliciting any undesirable effects in these cells, or inducing any undesirable local or systemic responses in the host.
- *Mechanical strength*—Scaffolds that are used as ECM replacement of damaged myocardium should have the mechanical properties to contain and protect the seeded or recruited cells and maintain their structure under mechanical perturbations existing during cultivation and at implant site. At the same time, the scaffold mechanical properties should be compatible with the host tissue to allow its integration without interfering with the normal function of the organ.
- *Biodegradation/bioresorption*—Ideally, the scaffold should disappear from the host when tissue regeneration has been accomplished and normal function is restored.
- *Scaffold fabrication*—Ideally, this process should use safe reagents which do not affect material properties, such as its cell recognition motifs.
- *Scaffold internal morphology*—When used as scaffolding for cells, the matrix should be porous with interconnecting pore structure and pore size larger than 50 μm , to enable cell–cell interactions and support vascularization after implantation.

Polymers commonly used for scaffold fabrication can be categorized by their source origin (natural or synthetic) and by their chemical structure (peptides/proteins, polysaccharides, polyesters, and others). [Table 18.2](#) lists the main materials used in cardiac tissue engineering strategies, as components in cellular constructs or standalone biomaterial treatments, and presents their main characteristics according to the design criteria listed earlier, namely, biocompatibility, mechanical strength, biodegradability/bioresorption, fabrication, and *internal morphology*.

Scaffolds can be fabricated in different shapes, sizes, and internal structure (porosity, pore size, and architecture of the pore structure (isotropic or anisotropic)). In tissue engineering of patches, either with seeded cells or without cells, the two main scaffolds in use are hydrogels and macroporous solid scaffolds.

18.6 Biomaterials as vehicles for stem cells or bioactive molecule delivery after MI

18.6.1 Stem cell delivery

The use of biomaterials as a platform or vehicle for cell delivery into the infarcted myocardium can potentially reduce or eliminate the obstacles seen in cell suspension transplantation, such as low engraftment, survival, and unpredictable retention. The biomaterials can protect the implanted cells

Table 18.2 Main biomaterials applied in cardiovascular tissue engineering.

Type	Material	Biodegradability/ control over process	Cross- linking	Mechanical strength	Cell adhesion	Biocompatibility/ Immunogenicity
Natural proteins	Collagen	Biodegradable; poor	Chemical	Med-low	Good, cross- linking Reduce	Med-immunogenicity after cross-linking
Natural- polysaccharides	Gelatin	Biodegradable; poor	Chemical	Low	Good	Low immunogenicity
	Fibrin	Biodegradable; good	Enzymatic	Med-low	Good	Nonimmunogenic
	Alginate	Bioerodable matrix; good	Physical	Low	Poor	Nonimmunogenic
	Chitosan	Biodegradable; good	Physical, chemical	Med-low	Poor	Low immunogenicity
Synthetic	Hyaluronan	Biodegradable; poor	Chemical	Low	Good	Nonimmunogenic
	PIPAAm	Nonbiodegradable; bioerodable	Chemical	NA	Good (temperature- dependent)	Biocompatible; NA
	Self-assembling peptides	Biodegradable; NA	Physical	NA	Good	Nonimmunogenic
Natural	Polyesters (PCL, PLA, PGA, and copolymers)	Biodegradable; good	Chemical	High	Poor	Med-low-no immunogenicity
	Decellularized ECM	Biodegradable, poor	Chemical	Med-low	Good	Nonimmunogenic, possible pathogen transmission

PCL, *poly(ϵ -caprolactone)*; PGA, *polyglycolic acid*; PIPAAm, *poly(N-sopropylacrylamide)*; PLA, *polylactic acid*; NA, *not available*.

from the aggressive environment after acute MI. In addition, biomaterials could provide various biochemical and biophysical cues to enhance cell survival, induce angiogenesis at infarct, and direct and control stem/progenitor cell differentiation, by implementation of controlled delivery mechanisms for growth factors, integration of specific cell–matrix interactions, and more (see Chapter 12 **Controlled Release Strategies in Tissue Engineering**).

Several studies have examined the use of biomaterials (natural and synthetic, in hydrogel or scaffold forms) as vehicles for stem cell delivery to the infarct. Due to relatively easy isolation methods, potentially autologous nature, and promising therapeutic potential, most of the studies are focused on biomaterial-assisted delivery of MSCs.

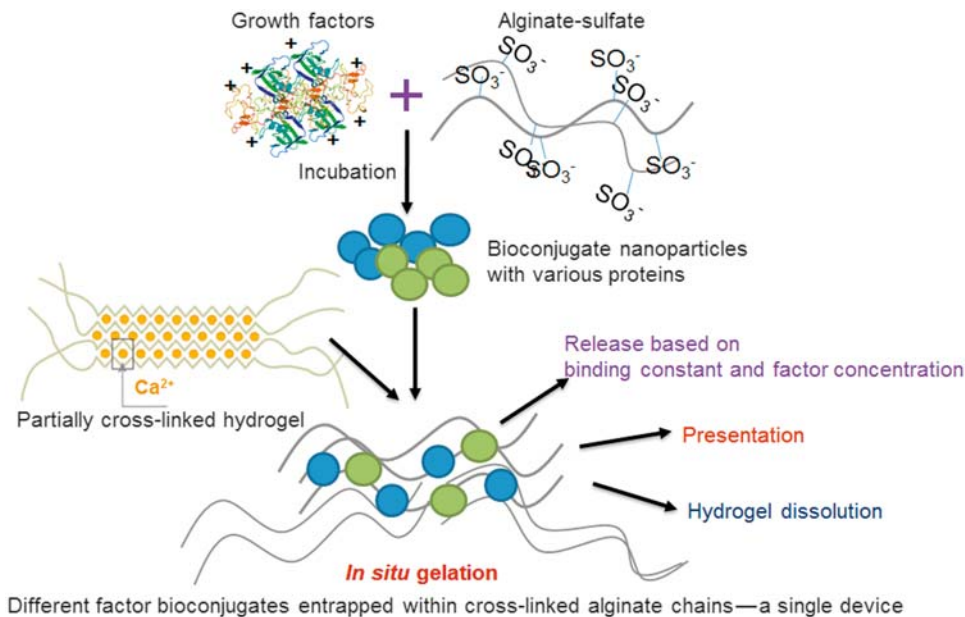
18.6.2 Delivery of bioactive molecules

Limited success in clinical trials of stem cell transplantation, the lack of true regeneration, together with emerging evidence pointing to **paracrine** effects (when substances secreted by the cell acting on adjacent cells) as the major mechanism of stem cell–based repair, lead researchers to examine an alternative cell-free strategy aimed at delivery of various bioactive therapeutic molecules (growth factors, cytokines, and stem cell mobilizing factors) to the infarcted heart. The variable effects exerted by these molecules cover almost every target in the regeneration strategies (see Chapter 12 **Controlled release strategies in tissue engineering**).

The systemic delivery of some of the growth factors was found to be beneficial for the restoration of cardiac function in animal models. However, data emerging from clinical studies have been less conclusive. The mixed results obtained with systemic cytokine or growth factor administration are also accompanied by numerous safety concerns and side effects. These include an increased incidence of **restenosis** (reoccurrence of stenosis in a blood vessel or heart valve after it has been treated with apparent success), elevated blood pressure and viscosity, **arrhythmogenesis** (induction of an alteration in rhythm of the heartbeat either in time or force), and other potential detrimental effects. In addition, **systemic administration** (administration of a drug into the circulatory system) requires higher doses of the drug due to unspecific delivery, fast elimination, and extremely low protein stability in the blood. Moreover, most of these molecules have **pleiotropic** functions, namely, other functions than those for which the molecules were originally intended to, emphasizing the need for careful, local, and time-adjusted interventions.

Thus, the combination of bioactive molecules with biomaterials to achieve effective local and temporary delivery seems to be a very attractive option. In particular, injectable systems composed of biomaterial–bioactive molecule combinations offer an additional advantage to the scaffold-based systems, i.e., greater applicability in the clinical setting due to the less invasive route of delivery.

For example, an injectable system based on engineered alginate was developed to enable precise control over growth factors' release rate.¹ The alginate was engineered with affinity binding sites for heparin-binding proteins (HBPs) by sulfation of the uronic acid monomers. The binding of various HBPs to alginate–sulfate mimics an example from nature, where many growth factors, chemokines, and cell adhesion molecules, collectively known as HBPs, bind heparin and heparan sulfate via high affinity, specific electrostatic interactions with the low- and high-sulfated sequences in these GAGs.

**FIGURE 18.6**

The concept of injectable affinity-binding alginate biomaterial and mode of protein release and action. From *Ruvinov et al., Cardiac tissue engineering: principles, materials, and applications. In: Synthesis Lectures on Tissue Engineering; 2012.*

Combination of alginate–sulfate with pristine alginate represents a unique type of a novel **affinity-binding** alginate biomaterial, which is capable of controlling the presentation and delivery of multiple proteins, while retaining the properties and characteristics of the alginate hydrogel as an ECM-replacing material. The concept of affinity-binding alginate biomaterial and mode of protein release and action is described in Fig. 18.6.

An injectable form of this biomaterial system was tested in an acute MI model in rats. The system was easily delivered into the infarcted heart, where it created a hydrogel capable of sustained delivery of multiple factors inducing myocardial regeneration. In this study, it was found that the sequential delivery of IGF-1 and hepatocyte growth factor reduced scar fibrosis, increased scar thickness, and prevented infarct expansion, compared to controls. Furthermore, angiogenesis was induced, and cell apoptosis was reduced at the infarct.²

18.7 Bioengineering of cardiac patches, in vitro

The replacement of a large scar tissue after MI and the corrections of congenital heart malformations, such as **septal defects** (defects in the septum, the wall that separates the two sides of the heart), require the transplantation of a fully developed cardiac tissue graft prepared in vitro. The engineered cardiac patch should be thick and has to display the functional and morphological properties of the native cardiac muscle. Once integrated into the heart, the cardiac patch must develop a **systolic force**, the force

that blood exerts on the artery walls as the heart contracts, withstand **diastolic load**, the load that is caused by relaxation and dilation of the chambers of the heart during which they fill with blood, with appropriate compliance, and form an electrical and functional **syncytium** (an interconnected mass of fibers) with the host myocardium.

18.7.1 Strategies for in vitro cardiac patch engineering

Three main strategies for in vitro cardiac patch reconstruction have been developed and are based on seeding the cardiac cells in scaffolds in the form of hydrogels, polymer layers, or macroporous scaffolds (Fig. 18.7).

A hydrogel is a network of polymer chains that are water insoluble, sometimes found as a colloidal gel in which water is the dispersion medium. Hydrogels are superabsorbent (they can contain over 99% water) natural or synthetic polymers. In cardiac cell entrapment strategy, cells are mixed with a liquid form of the biomaterial, followed by its solidification to create a mixed 3D cell hydrogel. This approach is also widely used in 3D bioprinting of cardiac patches.

Another approach for in vitro cardiac patch construction is the **cell sheet–based approach**, in which CM monolayers are assembled to form multilayered heart muscle constructs. As shown in Fig. 18.8, CM layers were initially formed by cultivating isolated cardiac cells on cell culture surfaces, grafted

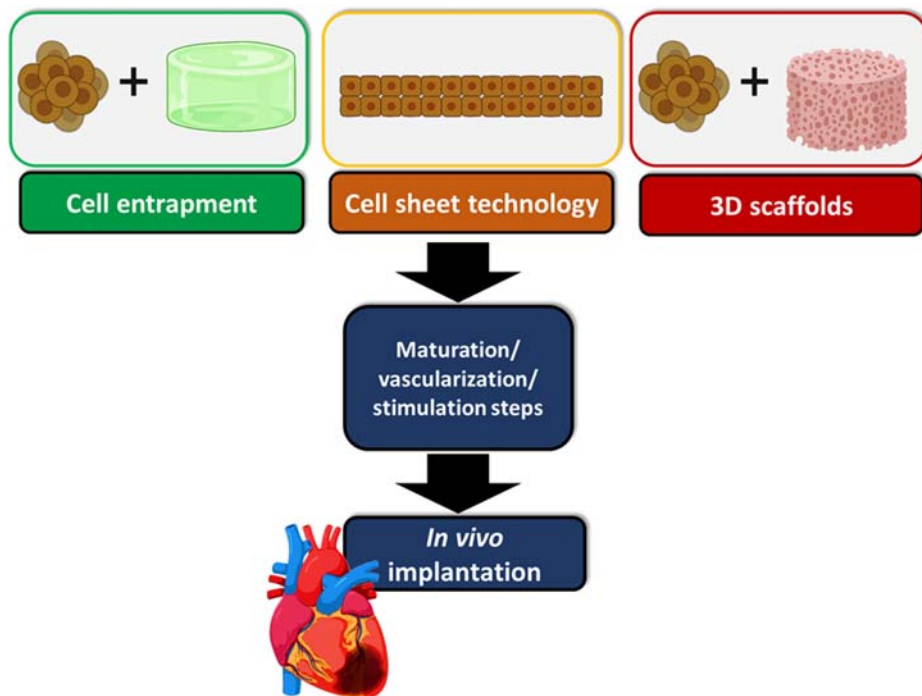
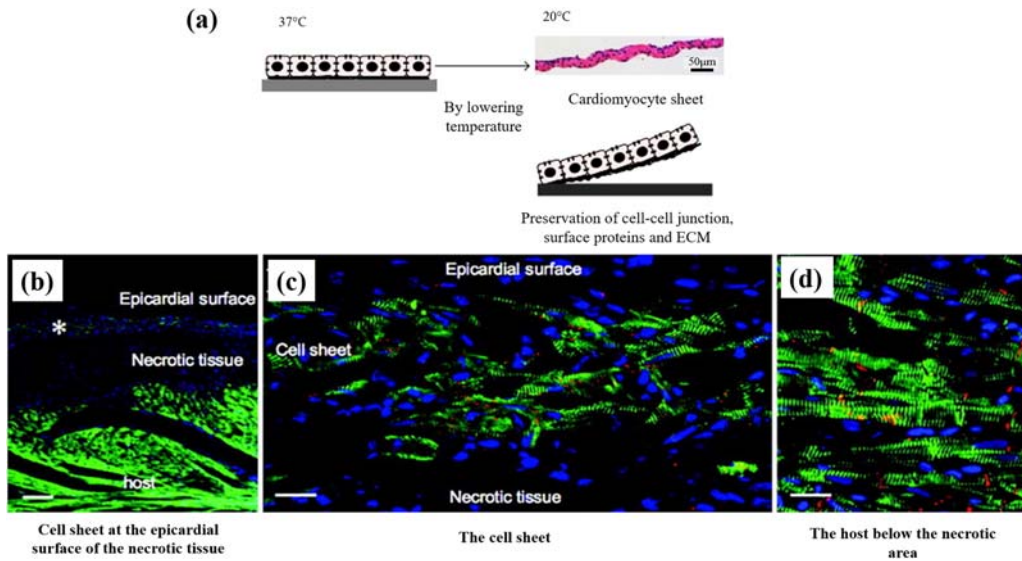


FIGURE 18.7

Strategies for reconstructing the cardiac patch. Created with BioRender.com.

**FIGURE 18.8**

Cell sheet-based cardiac tissue engineering (scale bar: 20 μm). From *Ruvinov et al., Cardiac tissue engineering: principles, materials, and applications. In: Synthesis Lectures on Tissue Engineering; 2012.*

with a temperature-responsive polymer. Later, confluent cell monolayers could be detached from the surface as a cell sheet, simply by reducing the temperature, without any enzymatic treatments. The released cell layers (2D) were overlaid one on top of the other (Fig. 18.8a), forming a multilayered 3D tissue that began to pulsate simultaneously. Laser confocal microscopic view of the grafted cell sheet and host heart, stained for the contractile protein, α -actinin (green), the gap junction protein, anticonnexin (Cx)-43 (red), and DAPI (blue) to stain the nucleus can also be seen in Fig. 18.8b. An α -actinin-positive cell sheet at the epicardial surface of the necrotic tissue is marked with (*) (Fig. 18.8b). In the cell sheet, clear striation pattern of α -actinin and diffuse Cx-43 staining are seen (Figs. 18.8c and d).

An additional approach is based on preformed implantable scaffolds. In this approach, cardiac cells are seeded within preformed macroporous scaffolds. The scaffolds are generally prepared by cross-linking (chemical or physical) of a biomaterial solution into the desired shape, with a subsequent solidification step and/or drying/freeze-drying. This platform offers important advantages over cell entrapment during hydrogel fabrication, such as the ability to design and control the porosity of the scaffold prior to cell seeding and reduced exposure of the seeded cells to stress during mixing and molding of the scaffold. Furthermore, the predesigned scaffolds can direct the seeded cells to organize into the gross conformation of native cardiac tissue, for example, by influencing cardiac cell alignment in the porous structure.

One of the first reports on successful implementation of myocardial tissue grafts based on this approach is an implantation of cardiac cell-seeded porous alginate scaffolds into infarcted rat hearts.⁴ The seeded fetal rat cardiac cells retained viability within the scaffolds and within 24 h formed multicellular beating

cell clusters. Furthermore, some of the cells appeared to differentiate into mature myocardial fibers. The graft and surrounding area were populated with a large number of newly formed blood vessels, consequently leading to attenuation in LV dilatation and improved heart function.

The efficacy of this strategy was further proven with patches prepared from other materials, natural or synthetic, emphasizing the potential of this approach for myocardial repair.

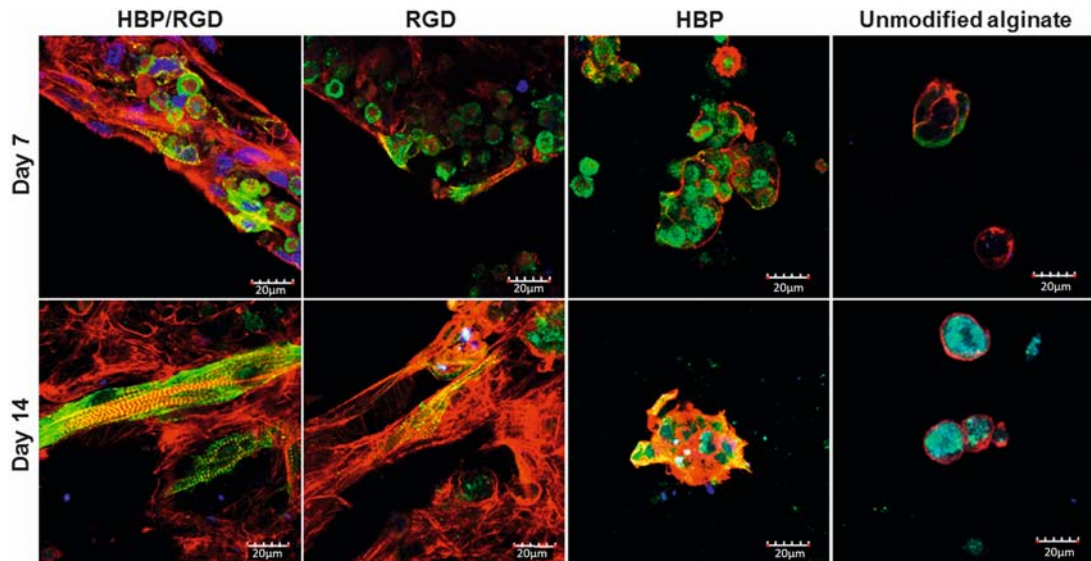
18.7.2 Biomimetic scaffolds and integration of cell–matrix interactions

Along with the use of ECM-extracted biological materials as scaffolds, which intrinsically retain signals important for cell–matrix interactions, major efforts have been invested in the intelligent design of novel synthetic materials bioinspired by the ECM interactions. These materials were created to provide the appropriate interface for cell interactions, including enhanced cell adhesion, growth, migration, and differentiation, while eliminating the potential hazards associated with the use of ECM-extracted materials.

An extensively used strategy applied to achieve such appropriate material surfaces has been by attaching ECM-derived synthetic peptides to the scaffold. The most commonly used peptide is the sequence Arg-Gly-Asp (RGD), the signaling domain derived from fibronectin and laminin. The RGD peptide mediates cell adhesion and signaling via binding of various ECM proteins to integrin receptors on the cell surface.

For example, RGD peptide was covalently attached to alginate, which is inert to cellular interactions, for the fabrication of engineered cardiac tissue in vitro.⁵ The RGD peptide decoration of alginate scaffolds accelerated in vitro cardiac tissue regeneration and contributed to better preservation of the tissue in culture. Moreover, the integration of an additional molecular signal provided by HBPs promoted the formation of improved cardiac tissue in vitro.⁶ Cardiac tissue engineering of neonatal rat cardiac cells cultivated within macroporous alginate scaffolds bearing the two peptides, RGD and HBP, was compared to those formed in a single peptide-attached or in unmodified alginate scaffolds. After 14 days, the characteristic striated fiber organization was observed mainly in the RGD/HBP-attached cell constructs, while in HBP-attached and unmodified alginate cell constructs no such structures were observed. Fig. 18.9 illustrates cardiac cell organization on days 7 and 14 postcell seeding (CMs are positive for α -actinin (green), while all cells are stained for F-actin (red) and nuclei (blue) scale bar: 20 μ m). These results emphasize the importance of integration of complementary signals into 3D matrices for the successful formation of an engineered cardiac tissue in vitro.

A common scaffold fabrication strategy is based on electrospinning (a polymer process technique capable of producing continuous fibers with diameters ranging from microns to nanometers in size) in which three-dimensional fiber structures can be made with high porosity, resembling the fibrous proteins in natural ECM. As mention above, the matrix should be porous with interconnecting pore structure and appropriate pore size, to enable cell–cell interactions and support vascularization after implantation. This principle was interestingly illustrated when electrospun PCL (poly(ϵ -caprolactone)) scaffolds with increasing average fiber diameters (3.4–12.1 μ m) were seeded with human venous myofibroblasts. During 3 days of culture, cell migration into the nanofiber scaffolds increased with fiber diameter. Furthermore, unrestricted cell infiltration was observed only in scaffolds with an average fiber

**FIGURE 18.9**

Integration of multiple cell–matrix interactions into alginate scaffolds promotes cardiac tissue organization. *From Ruvinov et al., Cardiac tissue engineering: principles, materials, and applications. In: Synthesis Lectures on Tissue Engineering; 2012.*

diameter proportional to the length of a cell and emphasizes that the optimal electrospun scaffold is cell specific, due to cell size differences between cell types.⁷

18.7.3 Perfusion bioreactors and stimulation patterns

In order to create relevant cardiac patches *in vitro* in terms of size, oxygen diffusion limitations have to be overcome. This has been accomplished by the use of perfusion bioreactors. As discussed in Chapter 13 **Bioreactors: enabling technologies for research and manufacturing**, the application of perfusion, by forcing the medium into the entire cell-seeded scaffolds, enables efficient mass transfer of oxygen and other soluble factors throughout the entire developing tissue, in a manner similar to the role of the vasculature in tissues.

In addition, physical cues are required to improve the contractile properties of engineered cardiac patches. Under cultivation, mechanical stimulation can be achieved by auxotonic stretch unidirectional cyclic or via contraction induced by electrical field stimulation. Thus, combining mechanical stimulation and culture medium perfusion may provide the means to engineer thick and contractile cardiac patches.

Mechanotransduction (biochemical response of cells to mechanical stimulation) is a fundamental process in any living cell. The ability of cells to convert mechanical forces into biochemical regulatory information is crucial for normal physiology and pathological processes in many tissues. Thus, the ability of various engineering efforts to recreate these signals *in vitro* is critical for the formation of functional cardiac patches.

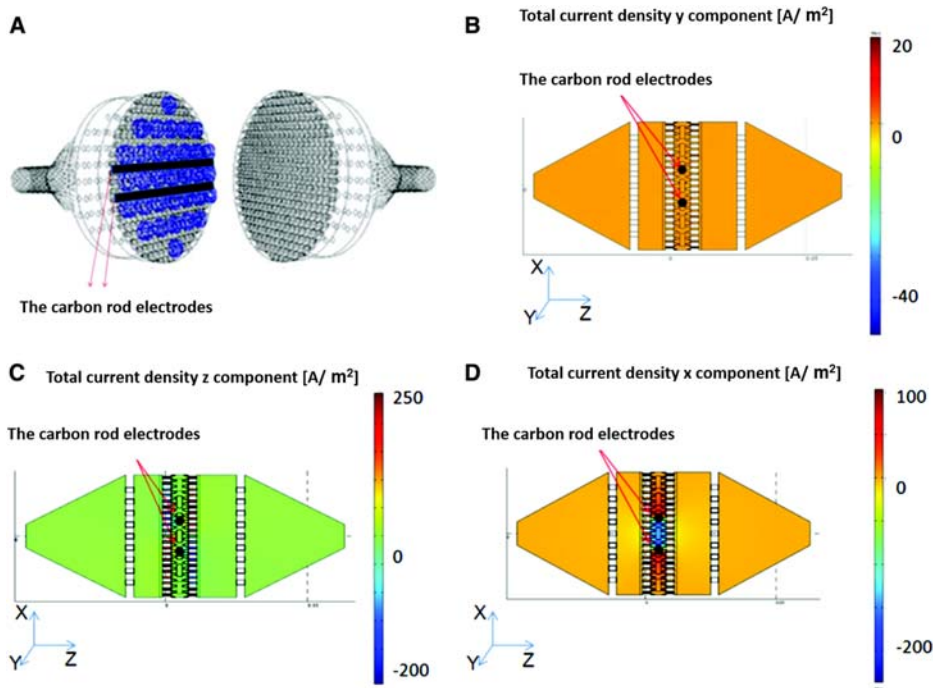
CMs employ numerous mechanisms to sense external forces and translate them into biochemical signals. In such a way, CMs can respond to elevated loading conditions, in an attempt to adapt to new mechanical demands and restore wall stress to normal levels, with an increase in cell size, associated with transient and persistent molecular changes, a process generally referred to as **cellular hypertrophy**. This process and biomechanical dynamics are important in maturation and organization of native cardiac tissue.

Nowadays, a leading approach in mechanostimulation of the cardiac constructs is via stimulation within a perfusion bioreactor during construct cultivation. The advantages of such bioreactor as a mechanostimulator are obvious; the interstitial medium flow within the porous cell construct creates a frictional force on the surface of the cells, termed **shear stress**.

Mammalian cells respond to fluid shear stress in different ways, for example, by enhancing proliferation or changing morphology and organization. For example, when a perfusion bioreactor with cardiac constructs was operated at a shear stress of 0.6 dyn/cm^2 , activation of the extracellular signal-regulated kinase 1/2 signal transduction pathway was pronounced, followed by enhanced synthesis of the contractile proteins (troponin-T and sarcomere α -actinin) and cell–cell interaction proteins (Cx-43 and N-cadherin). More importantly it was shown that long-term cultivation under such a pulsatile flow regime promoted organization of thick, highly organized cardiac tissue, resembling the native heart.⁸

From the moment it is formed, the heart acts as a sequential contracting syncytium. Although each CM is able to contract spontaneously, the overall heart function is controlled by a group of specialized **pace-maker cells**. These cells stimulate the generation of cardiac pulse and trigger synchronous contraction. In cardiac tissue engineering, the addition of exogenous electrical stimulation contributes to the reconstruction of an appropriate environment for tissue regeneration by mimicking the pacemaker activity during heart tissue development.

Various electrode materials, amplitudes, and frequencies of stimulation were tested to determine the conditions that are optimal for cardiac tissue engineering under static conditions. Carbon electrodes, exhibiting a high charge injection capacity and producing cardiac tissues with good structural and contractile properties, are thus used in tissue engineering studies. Specifically, engineered cardiac tissues stimulated at 3 V/cm amplitude and 3 Hz frequency had a high tissue density, a high concentration of cardiac Troponin-1 and Cx-43 and a well-developed contractile behavior.⁹ Electrical stimulation has been also implemented in tricell constructs (CMs, ECs, and fibroblasts) resulting in the formation of vascularized cardiac tissue.¹⁰ Electrical stimulation setups can be integrated into perfusion bioreactors. Fig. 18.10 shows an example in which carbon rod electrodes were inserted between two scaffold-mesh holders of a bioreactor. An illustration of the 3D configuration is presented in Fig. 18.10a (the carbonelectrodes in black and the cell constructs in blue). The current densities in the y, z, and x directions (1 V between the electrodes) are illustrated in Fig. 18.10b to Fig. 18.10d, respectively. In the presented system, a successful stimulation of the cell construct in a static cultivation mode was achieved at 6 V with a current density of 74.4 mA/cm^2 , while in the bioreactor with the carbon rod electrodes, 5 V was sufficient to achieve the same current density. Already after 4 days under a continuous electrical stimulus in perfusion bioreactor, cell elongation and striation in the cell constructs were clearly detected, as well as an enhanced expression level of Cx-43.

**FIGURE 18.10**

Electric field stimulation integrated into perfusion bioreactor for cardiac tissue engineering. From Ruvinov et al., *Cardiac tissue engineering: principles, materials, and applications*. In: Synthesis Lectures on Tissue Engineering; 2012.

18.8 Vascularization of cardiac patches

When designing functional and clinically relevant cardiac constructs, engineering techniques must integrate effective vascularization methods. Here, we discuss two approaches to overcome diffusion limitations in engineered cardiac tissues.

18.8.1 Prevascularization in vitro

Much effort has been invested into identifying the ideal cell combination for engineering a functional cardiac tissue. A thick graft seeded with CMs only requires support from host vasculature and is likely to undergo necrosis, due to both the extended lag period preceding infiltration of host vessels and the oxygen diffusion limitation. Incorporation of ECs within the graft enables in vitro generation of vascular-like networks, which can become functional and **anastomose** (interconnect of blood vessels) with host vasculature postimplantation.

For instance, a triculture of human embryonic stem cells—derived CMs, human umbilical vein endothelial cells (HUVECs), and mouse embryonic fibroblasts seeded on poly-L-lactic-acid/poly-lactic-co-glycolic-acid scaffolds resulted in the organization of vessel-like networks. Additionally, this combination enhanced cardiac differentiation in vitro, functionally manifested by spontaneous

synchronous contractions.¹¹ Upon implantation of the spontaneously contracting patches into the anterior wall of the LV, the vessel networks which originated from the preseeded ECs were perfused and anastomosed with host vasculature and promoted tissue survival and integration.¹² Moreover, sequential seeding of an EC-fibroblast 2-day coculture followed by seeding CMs on the scaffold resulted in the formation of compact contractile cardiac organoids, bearing elongated, Cx-43-expressing CMs, with higher viability when compared to the control.¹³

18.8.2 Prevascularization in vivo

Patch vascularization can be induced in vivo by patch preimplantation in an animal model that serves as bioreactor-like environment in which host vessels penetrate to the patch. Subsequently, the patch is detached from the site of origin and transferred to a new location to anastomose with host vasculature (see Chapter 14 **Vascularization, survival, and functionality of tissue-engineered constructs**).

A cardiac patch was constructed by seeding rat neonatal CMs onto an affinity-binding alginate scaffold supplemented with IGF-1, stromal cell-derived factor-1, and vascular endothelial growth factor. Vascularization was induced in vivo by first implanting the cardiac patch onto the rat adipose tissue for 1 week followed by implementation in a rat MI model. Four weeks postimplantation, the patches were well integrated with host myocardium, bearing troponin-T- and Cx-43-expressing cells with a defined cardiac structure. Significant cardiac function improvement and LV remodeling were observed. Moreover, the patches exhibited electrical coupling with host myocardium, as well as a rich vasculature.¹⁴

In vivo prevascularization can also be achieved by an **arterio-venous (AV) loop**, an anastomosis between an artery and a vein that shunts arterial blood into the vein. Transferring an AV loop with the desired scaffold to an enclosed implantation chamber creates an isolated microenvironment in vivo, which is connected to the living organism only by means of the vascular axis. The scaffold is then perfused by the AV sprouting vessels, yielding a well vascularized tissue graft, which can later be implanted. This approach has been applied with rat neonatal CMs seeded on Matrigel in a polycarbonate chamber.¹⁵

Overall, incorporation of functional vessels, whether performed in vitro or in vivo, facilitates scaffold vascularization, which in turn can form a massive vascular graft that can be applied to replace damaged tissue.

18.9 Three-dimensional bioprinting of vascularized tissues and components of heart

Three-dimensional (3D) bioprinting is currently emerging as a powerful strategy for engineering functional tissues. Bioprinting is defined as a computer-aided transfer process for simultaneous writing of living cells and biomaterials with a prescribed layer-by-layer stacking organization to fabricate bioengineered constructs for tissue engineering (TE). The preference of 3D bioprinting over previous strategies in TE stems from the high resolution and precise control that it provides. Practically, all the principles of cardiac TE that have already been mentioned in this chapter can be applied with relative ease by 3D bioprinting to create anatomically relevant 3D cardiovascular tissues (see Chapter 11

Scaffold design and fabrication). Pressure-based extrusion is one of the common methods to 3D print cardiovascular tissues at a moderate cost. This approach uses a differential pressure, generated by a syringe pump or a pressure reservoir upstream, to drive the material through a nozzle. Hence, most materials with appropriate viscosities for printing can be used, including hydrogels and direct printing of cells, enabling the fabrication of cellular microfluidic channels, cellular cardiac patches, and heart-like structures. These applications are described in the following sections.

18.9.1 3D bioprinting strategies for generation of a vascular network

The simplest 3D-printed vessel-like structures to be fabricated and investigated are arrays of uniaxial channels. The strategies for printing of these channels generally can be divided into two groups: direct printing of cellular microfluidic channels and printing of sacrificial materials to generate channel networks within engineered tissue constructs.

Direct printing of cellular microfluidic channels

Vascular networks can be designed by a one-step hydrogel printing approach, without the need for pre/postprocessing. Subsequently, the printed microfluidic channels can be embedded within a bulk hydrogel. A key player in this approach is a coaxial nozzle, which is utilized for the fabrication of cell-laden microfluidic channels in a broad range of dimensions. The coaxial nozzle allows the cross-linking of hydrogels during the printing process by generating a controlled interface between hydrogels solution and cross-linker solutions.

Two main polysaccharide solutions have been utilized by researchers for this application: sodium alginate and chitosan, due to their relatively rapid physical cross-linking by calcium chloride and sodium hydroxide, respectively. However, alginate hydrogel was proven to have relatively better mechanical and structural integrity compared to that of chitosan. Specifically, as shown in Fig. 18.11, the alginate solution is dispensed through the outer tube of a coaxial nozzle, while the calcium chloride solution is

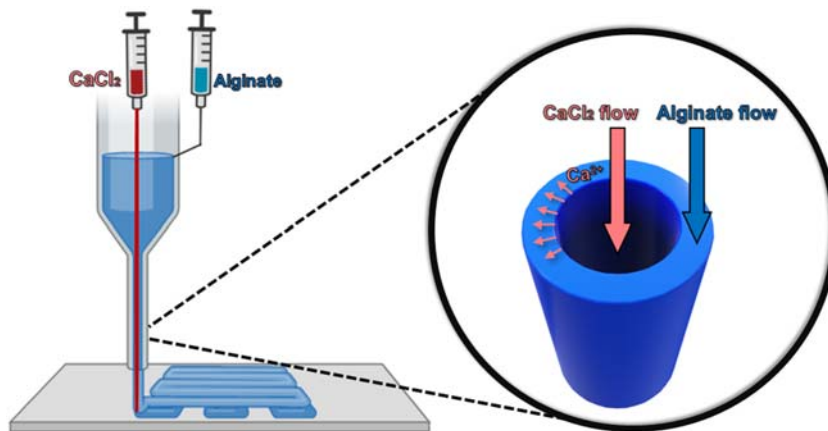


FIGURE 18.11

A schematic presentation of coaxial nozzle-assisted 3D bioprinting system for the fabrication of alginate structure with built-in microchannels.

dispensed through the inner tube of the coaxial nozzle. By this set-up, the two solution flows contact each other, Ca^{2+} ions diffuse into the alginate solution, and instantaneous gelation occurs, yielding a tubular gel with a hollow channel, while the cross-linker flowing through the inner tube forms the hollow core.

By this fabrication approach, the formed hollow channel size can be controlled by changing the flow rate of the given solutions, the solution concentrations, and the nozzle diameter. Specifically, increasing the cross-linker flow rate generates more tension on the channel wall in the radial direction, expanding the channels in this direction and increasing both core and channel diameter. In addition, the diffusion rate of Ca^{2+} ions is influenced by the alginate concentration and hence affects the channel diameter.

Generally, the direct printing of perfusable microfluidic channels by a coaxial nozzle is a simple and fast method. However, it is restricted to a few specific biomaterials and has only limited resolution. Furthermore, a major challenge in the direct printing of microchannels is their tendency to fold or collapse under their own weight. Hence, in fact, extrusion printing is often not freeform in all three dimensions. A common approach to overcome this obstacle is printing in the presence of support materials, which are sacrificed via external stimuli after printing.

Channel networks fabrication methods based on sacrificial technology

In the context of channel network fabrication, sacrificial materials can be used as a primary template of strands that encased in a second bulk material. Selective removal of the sacrificial material yields a channel network in the bulk material which retains the architecture of the original template.

In general, 3D sacrificial materials for channel bioprinting should possess two important characteristics addressing seemingly opposing requirements: sufficient mechanical stiffness to physically support the fabricated structure in three dimensions and the ability to dissolve rapidly while maintaining biocompatibility in the presence of living cells. Practically, a wide variety of biomaterials can serve as sacrificial materials by being removed through an appropriate mechanism after printing.

For example, the thermo-sensitivity of gelatin makes it a potential candidate for application as a sacrificial material. HUVEC-laden gelatin ink was printed as a straight strand, at room temperature, on polymerized collagen layers, within a flow chamber. Next, more collagen layers were printed to cover the gelatin pattern. The construct was then incubated at 37°C for complete collagen gelation and conversely, to liquefy the gelatin. During the incubation, HUVECs within the gelatin sank down and attached to the inner surface of the channel; hence, the inversion of the construct was necessary to allow cell attachment to both bottom and top surfaces of the channel. Following this step, for dynamic tissue culture under physiological shear, the flow chamber was connected to a dispensing pump applying a gentle media flow for washing out the gelatin and to obtain a perfused vascular channel. Under this condition, the channels experienced an endothelium straightening and cell elongation, whereas the proliferation was suppressed. Finally, the resulted vascular channel had a perfused open lumen, fully covered with ECs.¹⁶

However, anatomically relevant blood vessels have three layers characterized by different cellular components, stiffness, and functions. Due to limitations of resolution and scale, it is still difficult to print hierarchical constructs that mimic such layered structures.

18.9.2 3D bioprinting of heart-like structure

Owing to weak mechanical properties, hydrogel extrusion is generally limited to printing structures that are not greater than several millimeters in size or require only a low structural fidelity. Over the last few years, a new 3D bioprinting strategy of mechanically weak hydrogels was developed. This strategy is an extension of the sacrificial method and is based on printing bioink in a support bath composed of hydrogel microparticles. The support bath functions like a **Bingham plastic**, behaving as a rigid body at low shear stresses but flowing as a viscous fluid at higher shear stresses. Hence, while a needle moves through the bath, extrusion of bioinks against little mechanical resistance occurs, whereas the deposited structure is held in place within the bath. Subsequently, the hydrogel microparticles are removed in order to extract the printed construct (Fig. 18.12 illustrates this strategy).

Recently, bioprinting of a cellularized human heart structure utilizing the support bath strategy composed of alginate microparticles was demonstrated. This fabrication is a proof of concept of bioprinting complex anatomical structures from patient-derived mechanically weak bioink and includes iPSCs-derived CMs and ECs. The resolution achieved from printing in this support bath permitted perfusion of both the major blood vessels and the heart ventricles. However, this heart was miniaturized (with a height of 20 mm and diameter of 14 mm), and the cells contracted but did not work together to form a pumping ability (see State-of-the-art experiment for extension).

Although 3D bioprinting is useful for building complex constructs that can maintain the viability of cells, 3D bioprinting of myocardial tissue is mainly limited by: (i) the source of human cardiac cells, which will hopefully be addressed by iPSC, (ii) the limited resolution by which complex structures can be built, possessing a functional and hierarchical (on scales ranging from microns to millimeters) vascular system to be integrated with a host, and (iii) the limited methodologies for long-term cultivation for tissue maturation.

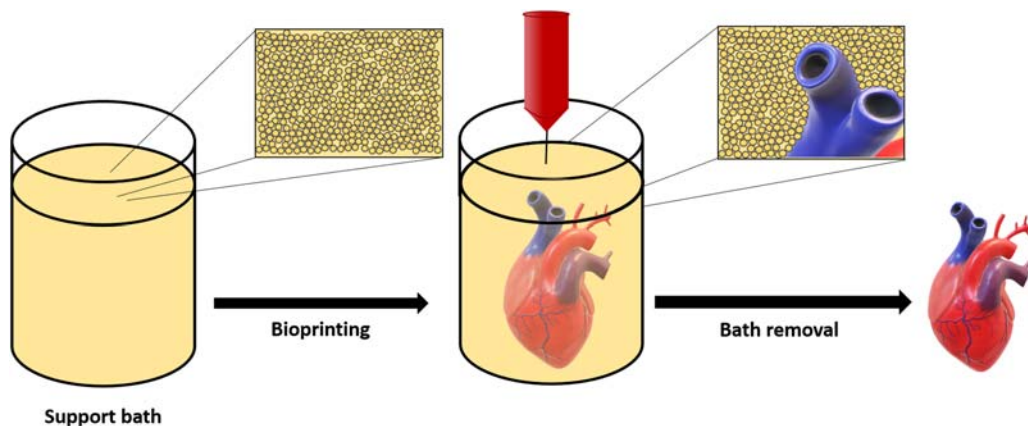


FIGURE 18.12

Two-step bioprinting process of heart-like structures.

18.10 Challenges for clinical application

Over the last decade, there have been huge advancements in cardiac tissue engineering which have rendered the possibility of building tissues to transplant into patients a tangible goal in the future. Transplantation of iPSC-derived CMs is now possible; however, to date, cardiac function and action potential remain suboptimal due to the transplanted cells being more neonatal-like and the electrical output being neither well integrated nor comparable to the hosts. Hence, full functional and electrical integration of cardiac patch with the host remains the largest obstacle.

Furthermore, there is a considerable disparity between the outcomes in preclinical studies of cardiac regeneration and the clinical trials of cardiac regeneration in humans. One explanation could be the differences between species, including the molecular basis of disease, physiology, and anatomy, which might account for translational failures. Another possible explanation is insufficient delivery of therapeutic factors to the target sites. As discussed above, this challenge may be addressed by utilizing biomaterial-based delivery systems, which can provide a scaffold that can improve the therapeutic effects of both cellular and acellular approaches.

Therefore, in order to repair the human heart, the limitations of preclinical studies of cardiac regeneration and the difficulties of clinical translation must be addressed.

18.11 Future perspective

The urgent need for cardiovascular replacement and regeneration therapies has led to a diversity of cardiovascular tissue engineering strategies. Advancements in stem cell technology, iPSC derivation, and effective cardiac differentiation and purification techniques offer cell sources suitable for the engineering of human heart tissues. Continuously evolving biomaterial schemes, novel polymers, and bio-inspired and “smart” materials, together with scaffold microfabrication advancements, bioreactor design, and effective maturation/stimulation regimens, offer a list of powerful tools to achieve optimized and long-term functioning grafts.

Biomaterials, when appropriately designed, can be effective vehicles for cell delivery, to improve cell retention and function. Moreover, multiple bioactive molecules can be delivered by biomaterials in a sequential and predicted order to activate the different reparative and regeneration processes in the infarcted heart.

In cardiovascular graft bioengineering, we can already witness significant advancements in terms of graft composition and functionality. Near future progress will include better ECM mimicking, controllable mechanical properties carefully matched to the target myocardial or vascular tissue, introduction of bioactive, dynamically responsive and bioinspired materials, effective maturation and vascularization techniques, and development of tools for high-throughput and quantitative assessment of graft quality.

We expect that in the following decade extensive and hard work of many dedicated and creative researchers will result in significant technological advancements achieved on the basis of already established principles laid in the chapter, and the concepts for new and maybe even more powerful strategies will be introduced. Such developments will offer true and effective alternatives to current palliative clinical therapies and will become a state-of-the-art treatment option for patients with cardiovascular disease.

Summary

- Low regeneration rate mainly currently applied palliative therapies, and low availability of donor tissues defines the urgent need for the development of new restorative strategies for cardiovascular repair and regeneration.
- Cardiovascular tissue engineering relies on the powerful combination of cells, biomaterials (natural or synthetic), and bioactive molecules (stem cell products or their derivatives).
- Biomaterials can be used as a platform or vehicle for cell delivery into the infarcted myocardium, and improve engraftment, survival, retention, and function.
- The “paracrine hypothesis” of stem cell action strengthens the idea behind selective delivery of bioactive molecules; the combination of bioactive molecules with biomaterials allows achieving effective local and sustained delivery with spatial and controlled distribution of the desired agent to induce multiple regenerative processes.
- The replacement of a large, damaged tissue requires the transplantation of a fully developed cardiac tissue graft prepared in vitro. Three main strategies for in vitro cardiac patch reconstruction have been developed, based on the combination of cardiac cells and hydrogels, polymer layers, or preformed macroporous scaffolds.
- Generation of human CMs from pluripotent stem cells has allowed bioengineering of human cardiac grafts for the first time.
- Development of perfusion bioreactors and various stimulation patterns (mechanical and electrical) can promote cell organization and graft maturation.
- Integration and survival of thick tissue constructs rely on effective graft vascularization. Several in vitro (incorporation of endothelial cells) and in vivo (host prevascularization) strategies have been suggested to achieve this goal.
- Current advancement in biomaterials and emerging 3D printing techniques provide a remarkable possibility to create anatomically relevant 3D cardiovascular tissues.

Classical experiment

The introduction of a microvascular network is particularly required in cases of generating thick constructs of highly metabolically active organs like the heart. One of the strategies which were proposed for myocardium engineering combines guided self-assembly of endothelial cells and 3D bioprinting.

For 3D printing, human umbilical vein endothelial cells (HUVECs) encapsulated within GelMA-alginate-photoinitiator bioink were dispensed through an internal needle of a co-axial nozzle. CaCl_2 solution was simultaneously dispensed through the outer sheath flow, resulting in physical cross-linking of the alginate. Subsequently, permanent chemical gelation was achieved by a light explosion of the formed microfibers for photo-crosslinking of the GelMA component.

Intrinsic polarization tendency of HUVECs to stay at liquid–matrix interfaces, as well as the high availability of nutrients and oxygen in this position, led to a gradual cell migration toward the peripheries of the microfibers at day 9 postprinting. Notably, the ionically cross-linked alginate component dissolved in culture medium and leached out from the microfibers in approximately 5–10 days, resulting in the formation of larger pores along the borders, since the alginate component was better cross-linked at the peripheries. This pattern further promoted

proliferation and spreading of the HUVECs, forming an organized layer of confluent endothelium at the microfiber’s borders within approximately 2 weeks of culture. Next, neonatal rat cardiomyocytes (CMs) were seeded and were allowed to adhere to the microfibers. This arrangement resembles the blood vessel walls.

In order to generate endothelialized myocardial constructs, the microfibers were further cellularized by neonatal rat CMs or human-induced pluripotent stem cells (hiPSC)-CMs.

Under perfusion conditions, the CMs aligned and tightly attached to the microfiber surface. Furthermore, highly synchronized and uniform beating appeared across the entire structure, whereas the beating rate lasted at approximately 60 bpm for up to 7–10 days.

Finally, cardiovascular drug screening was performed, utilizing a microfluidic perfusion bioreactor, generating endothelialized-myocardium-on-a-chip model which provided dose-dependent responses of both CMs and endothelial cells.¹⁷ The steps in Fig. 18.13 are as follows: Step 1: bioprinting of a microfibrinous scaffold using a composite bioink encapsulating endothelial cells. Step 2: formation of the vascular bed through migration of HUVECs to the peripheries of the microfibers. Step 3: seeding of

cardiomyocytes into the interstitial space of the endothelialized scaffold. Step 4: formation of engineered endothe-

lialized myocardium structurally resembling the native myocardium.



Schematics showing the procedure of fabricating endothelialized myocardium using the 3D bioprinting strategy. *From Zhang YS, et al. Bioprinting 3D microfibrinous scaffolds for engineering endothelialized myocardium and heart-on-a-chip. Biomaterials. 2016;110:45–59.*

State-of-the-art experiment

Three-dimensional (3D) bioprinting is an emerging technology that allows the assembling of both living cells and biological materials into an ideal complex layout to produce engineered tissue or organ. Various biomaterials and techniques have been utilized to bioprint biological constructs in different shapes, sizes, and resolutions. In order to engineer 3D-printed thick, vascularized, and perfusable cardiac patches that closely fit the patient's heart in terms of anatomy and immunogenicity, researchers have developed a smart personalized bioink. In this approach, a biopsy of fatty tissue was taken from patients and the cells and the extracellular matrix (ECM) were separated. Subsequently, the cells were reprogrammed to become pluripotent stem cells, and differentiated to cardiomyocytes and endothelial cells (ECs), whereas the ECM was processed into a personalized hydrogel. Next, each cell type was separately mixed with the hydrogel to form bioink. Basing on computerized tomography (CT) of a patient's heart, a 3D cardiac patch was designed by computer-aided design (CAD) software. In such a way, the patch dimensions and the blood vessel geometry were fitted to the host. However, CT cannot provide images of small blood vessels. Hence, smaller blood vessels were added to the basic vasculature design, according to Fick's second law (oxygen diffusion) and to Michaelis–Menten equation (consumption). Seven days after patch

transplantation between two layers of rat omentum, cell elongation and alignment were observed, and the contractility potential was indicated by massive striation.

Furthermore, small-scale cellularized human hearts (height: 20 mm; diameter: 14 mm) with major blood vessels were printed within a support medium with two distinct personalized bioinks, containing CMs or ECs. Printing in this support medium, composed of alginate microparticles in xanthan gum-supplemented growth medium, not only allows free-form printing but also can undergo safe enzymatic or chemical degradation for extraction, while maintaining high cell viability. After extraction, the left and right ventricles were injected with red and blue dyes, respectively, demonstrating the ability to perfuse the printed hearts, and illustrating their mechanical stability. Additionally, a homogeneous distribution of the CMs was observed one-day postprinting.

This research demonstrates the potential of 3D printing as an approach for engineering whole organs. However, limitations as cell expansion, long-term cultivation for tissue maturation, and precise printing of small-diameter blood vessels still remain.¹⁸ Steps in [Fig. 18.14a](#): The human heart CAD model. [Figs. 18.14b and c](#) a printed heart within a support bath. [Fig. 18.14d](#) after extraction, the left and right ventricles were injected with red and blue dyes,

State-of-the-art experiment—continued

respectively, in order to demonstrate hollow chambers and the septum in-between them. Fig. 18.14e 3D confocal image of the printed heart (CMs in pink, ECs in orange).

Figs. 18.14f and g cross-sections of the heart immunostained against sarcomere actinin (green). Scale bars: (c) 0.5 mm (d–f) = 1 mm, (g) = 50 μ m.

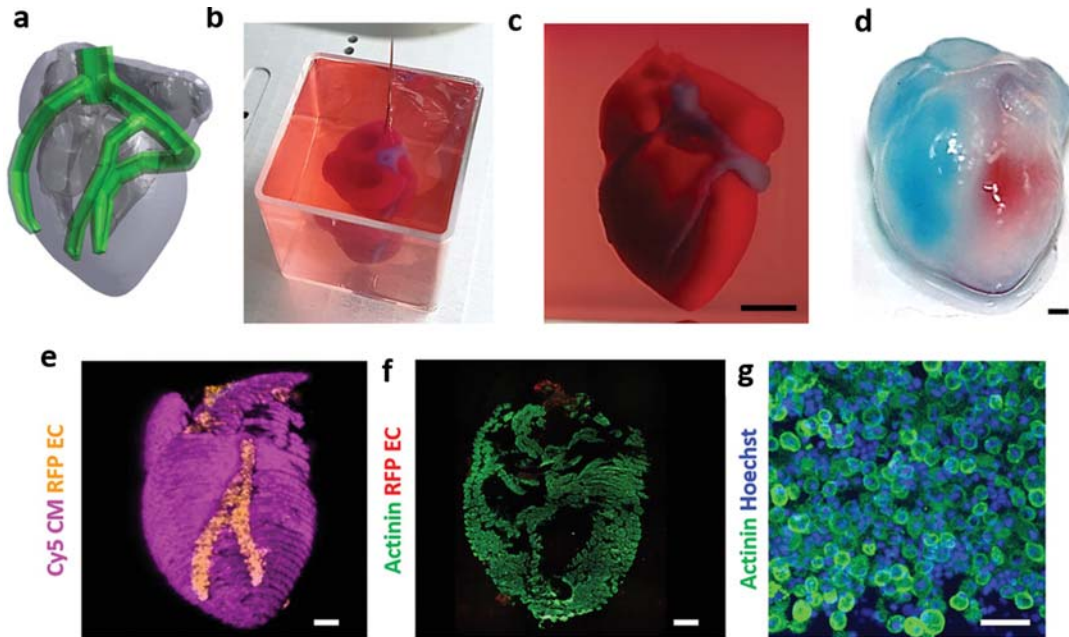


FIGURE 18.14

A printed small-scaled, cellularized, human heart. From Noor, et al. *3D printing of personalized thick and perfusable cardiac patches and hearts*. Adv Sci. 2019;6:1900344.

18.12 Recommended literature

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18.13 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
1. Which cell type is responsible for the heart-pumping action?
 2. What are the major ECM components in the myocardium?
 3. Define “infract expansion” in one or two sentences.
 4. Distinguish the five phases in the progression from healthy to the infarcted myocardium.
 5. List five major potential therapeutic targets and strategies to induce myocardial regeneration.
 6. What is the difference between cardiac and skeletal muscle?
 7. Name 4 cell sources for cardiovascular tissue engineering and regeneration.
 8. What benefits does the combination of bioactive molecules with biomaterials provide?
 9. Name three main strategies for in vitro cardiac patch construction.
 10. Describe shortly the cell sheet-based approach for in vitro cardiac patch construction.
 11. Which advantages do the preformed implantable scaffolds offer over the cell entrapment strategy?
 12. Give an example for a strategy used to achieve an appropriate material surface for cell interactions.

13. What cell type is positive for troponin-T, sarcomeric α -actinin, Cx-43, and N-cadherin staining?
 14. Define the term “Mechanotransduction.”
 15. What is the advantage of a perfusion bioreactor as a mechanostimulator?
 16. What kinds of stimulation can promote cell organization and cardiac graft maturation?
 17. Name two approaches to overcome diffusion limitations in engineered cardiac tissues.
 18. When alginate solution is dispensed through an outer tube of a coaxial nozzle and a calcium chloride solution is dispensed through the inner tube of the coaxial nozzle, how can the formed hollow channel size be controlled?
 19. What are the two important characteristics that 3D sacrificial materials for channel bioprinting should possess?
 20. What are the main limitations for the 3D bioprinting of myocardial tissue?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations:
1. Why would you consider is the reason behind using acellular grafts for cardiac TE?
 2. Which cell sources would you use for cardiovascular tissue engineering? Explain why.
 3. Describe the requirements of a cardiac tissue graft for the replacement of large scar tissue after MI.
 4. What would be your proposed strategy to induce cardiac patch vascularization?
 5. Suggest two reasons for the cultivation of cardiac patches within a perfusion bioreactor.
 6. Which experiments would you perform to evaluate the functionality of a cardiac graft?
 7. How would you achieve an in vivo prevascularized cardiac patch?
 8. What would be your proposed strategy for bioprinting small-diameter vessel grafts?
 9. Suggest an approach for engineering complex anatomical structures.
 10. What are the new possibilities for cardiac TE that the 3D bioprinting strategy facilitates?

Challenge-based learning

Fabrication of “sticky” biomaterials with antifibrotic and vascularization properties to treat myocardial infarction

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Cardiovascular diseases are the number one reason for nonaccidental deaths in the world. It is well documented that cardiovascular diseases worsen with a sedentary life style, sugar and fat diets, and stress. This can lead to fat and lipid deposits that can partially or totally block arteries like the coronaries in the heart. Sustained lack of irrigation to the heart muscle (myocardium) will alter heart function and lead to death if emergency medical care is not

provided. This event is called a myocardial infarction (MI) and can lead to inadequate and incomplete tissue regeneration and fibrosis. The long-term vision is to develop a therapeutical tool to regenerate the myocardium immediately after infarction before it progresses into fibrotic tissue.

Motivation and stakeholders

Inhibition of the fibrotic response after an infarction is shown to be effective in reducing the loss of cardiac tissue function. In addition to this, myocardium regeneration requires irrigation of the new tissue via the formation of new vessels. Novel biomaterial formulations are not only biocompatible but actively support the recruitment of cardiomyocytes and vasculature and inhibit the immune system. New solutions for minimally invasive technology to fix heart muscle defects after infarction should acknowledge

Continued

Challenge-based learning—continued

the requirements set out by the stakeholders such as the patients, interventional cardiologists, cardiovascular surgeons, biomedical engineers, and material scientists.

Problem definition

Novel tissue engineering cardiovascular strategies should aim to develop a material that can promote healing and neo-vascularization as it adheres to the walls of the beating and dynamic heart. This material should be in the form of a hydrogel that can be compressed in a canula and deployed to the injured heart site via catheter to minimize additional myocardium damage. Currently, no tissue engineering strategy is clinically available to be deployed using a catheter to mend fibrotic and avascular myocardium. A new generation of “sticky” biomaterials or hydrogels is required to recruit mesenchymal or progenitor-derived cells from the surroundings and the immune system (macrophages) to resolve fibrosis to ultimately replace it with healthy myocardium-like tissue.

Challenge

To design an effective “sticky” biomaterial or hydrogel with antifibrotic and vascularization properties that can be deployed on site noninvasively after myocardial infarction.

Learning framework

Reading the Cardiovascular Tissue Engineering chapter and related literature will help you to will help you to understand:

1. Heart and cardiovascular anatomy and physiology in humans.
2. Pathophysiology of heart injury such as myocardial infarction.
3. Current cardio-interventions and state-of-art treatments to identify and resolve myocardial infarctions.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

4. Current biomaterials used for cardiac tissue engineering.
5. Design criteria for biomaterials in cardiac tissue engineering.
6. Arthroscopic techniques to deliver biomaterials in situ.
7. Current strategies to produce sticky biomaterials.
8. Cellular and molecular to be targeted by the biomaterials.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

18.14 Glossary

Affinity binding is the strength of the binding interaction between a single biomolecule to its ligand/binding partner. It is the specificity of a ligand to strongly bind to its receptor.

Anastomose (Anastomosis) is the connection between two things that are normally diverging or branching, such as between blood vessels, leaf veins, or streams.

Arrhythmogenesis is a type of study where arrhythmia is induced and observed via electrocardiographic waveforms.

Arterio-venous loop is an in vivo tissue engineering strategy for generating axially vascularized tissues using the body as a bioreactor.

Autologous refers to cells or tissue obtained from the same individual.

Bingham plastic is a viscoplastic material that behaves as a rigid body at low stresses but flows as a viscous fluid at high stress.

Cardiomyocytes are the contractile cells of the heart.

Cell sheet–based approach refers to transplantation of bioengineered tissue layers onto damaged sites to minimize cell loss.

Cellular hypertrophy is an increase in size and mass of a cell, without proliferation. It should be differentiated from cellular hyperplasia where the number of cells increases.

Coronary heart disease is a term used for a variety of conditions consisting of the decrease of blood flow through coronary blood vessels which affects the structure and function of the heart.

Diastolic load is the amount of sarcomere stretch experienced by cardiac muscle cells, at the end of ventricular filling before the upcoming beat.

Endocardium is a thin, smooth tissue that makes up the lining of the chambers and valves of the heart.

Epicardium is the layer that covers the myocardium, which lays below the pericardium.

Extracellular matrix is a three-dimensional network consisting of extracellular macromolecules and minerals, such as collagen, enzymes, and glycoproteins that provide structural and biochemical support to surrounding cells.

Heart failure is the inability of the heart to properly pump blood around the body.

Hypertrophy is the increase in size of cells.

Infarct expansion is an alteration in the ventricular topography due to thinning and lengthening of the infarcted segment, which develops within the first few hours of the acute symptoms

Ischemic (ischemia) is the reduction or restriction of blood supply to tissues, preventing it from receiving enough oxygen and resulting in its dysfunction.

Mechanotransduction is the processes used by cells to sense mechanical stimulus and convert it to biochemical signals.

Myocardial infarction is the medical name of the heart attack, when oxygen supply to one or more areas of the heart muscle is restricted due to a blocked coronary blood vessel.

Myocardium is the muscle layer of the heart sandwiched between the endocardium and the epicardium.

Myogenic means originating in or produced by muscle tissue.

Pacemaker cells are heart cells that control the heart rate by creating rhythmic impulses.

Paracrine is a type of cellular communication in which a cell produces a signal to induce changes in nearby cells, altering their behavior. Factors are secreted into the immediate extracellular environment.

Pleiotropic is the ability of a single gene to produce or have multiple effects.

Pluripotent refers to cells that can give rise to different cell types that make up the body.

Restenosis is when a part of the artery that was previously cleared of blockage becomes narrow again.

Septal defects are abnormal openings in the wall that divides the right and left ventricles that appear during embryonic development.

Shear stress is the mechanical force sensed by cells exposed to flow.

Smooth muscle cells are spindle shaped cells containing centrally placed nucleus that form part of the nonstriated, involuntary muscle tissue lining hollow organs such as arteries, lungs, and bladder.

Syncytium is a multinucleate cell which can result from multiple cell fusions of uninuclear cells.

Systemic administration (of substances) is a route of administration of medication, nutrition, or other substance into the circulatory system so that the entire body is affected.

Systolic force is the force that blood exerts on the artery walls as the heart contracts to pump the blood to the peripheral organs and tissues.

Three-dimensional bioprinting is the utilization of 3D plotting techniques to combine cells, growth factors, and/or biomaterials to fabricate biomedical parts imitating natural tissue characteristics.

© Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Tissue engineering of organ systems

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19.1 Learning objectives

After reading this chapter you will be able to:

- Describe the commonalities and differences in techniques and concepts in organ tissue engineering.
- Recognize the importance of adequate mechanical properties of the matrix in organ tissue engineering.
- Recognize the requirement of using the human body as a “bioreactor” to allow final tissue differentiation.
- Know the limiting factors for successful in vivo implantation of in vitro engineered organs.
- Take note of the most recent biologic constructs in development and their current clinical applications.

It was very hard to live a normal life with spina bifida, my kidneys and bladder weren't working. I went through 16 surgeries and it seemed impossible to have a normal life. I went through kidney failure when I was 10, then this surgery came along and basically made me who I am today and saved my life.

Luke M. A patient who received a 3D printed bladder. 2013.

19.2 Introduction

Organ replacement, repair, or restoration of organ function is classically treated by organ or tissue transplantation to replace diseased, missing, or malformed organs. In cases where **congenital** malformation can lead to severe **organ agenesis; dysplasia and hypoplasia**; or anatomical, structural, and functional deficiencies, it might be necessary to replace the entire or part of the organ. In other cases, cancer, trauma, infection, inflammation, or **iatrogenic** injuries may lead to organ damage or loss and dictate eventual replacement. Yet three major problems are linked to human organ donation: tissue compatibility, tissue rejection, and donor shortage. Depending on the type of organ, donors and receivers need to be immunologically matched to minimize the risk of rejection, and the donor tissue needs to be of the highest quality to provide the best chance of obtaining long-term function, while for some vital and delicate organs such as pancreas, the number of available donor organs is scarce.

In some organ systems, these problems can be minimized by reconstruction with tissue from other organ systems when native tissue is lacking. For example, in the genitourinary tract, surgeons can use non-urologic tissues, such as intestinal or gastric segments, mucosa from several body sites, and skin with homologous tissues (e.g., cadaver fascia, cadaver, or donor kidney) to reconstruct vital structures. Other options include biopolymers (e.g., bovine collagen) or synthetic polymers (e.g., silicone, polyurethane or polytetrafluoroethylene, poly(glycolic acid) (PGA), poly(lactic acid), and poly(lactic-co-glycolic acid)), among others. These approaches are, however, often linked to complications, such as metabolic disturbances and even cancer, and rarely replace the entire function of the original tissues because of their inherently different functional parameters.

Although engineering of two-dimensional (2D) and very small three-dimensional (3D) tissue structures has been achieved, construction of true 3D functional tissue structures, acting as organ systems, is still a big challenge. This is even more so if the organ consists of a multitude of different cell types, each having a particular function and influencing each other. Such engineered constructs depend on very complex cell carrier matrices, often combined with biological factors, such as morphogens and extracellular matrix (ECM) components. This supports the respective cell types by promoting vascularization and allowing adequate nutritional support—a key issue in large 3D constructs.

In tissue engineering, biomaterials should act as artificial ECMs, and mimic biologic and mechanical functions of native ECM as much as possible (see [Chapter 5 Extracellular matrix as a bioscaffold for tissue engineering](#)). Biomaterials should also allow controlled delivery of bioactive factors (e.g., cell adhesion peptides, growth factors) and should offer a 3D space for the development of new tissues with appropriate structure and function.⁹ In other words, the optimal biomaterial should be **biocompatible**, promote cellular interaction and tissue development, and possess proper mechanical and physical properties and act as a delivery vehicle to control the localization of transplanted cells.

Three main types of biomaterials can be used for the engineering of bioartificial organs: naturally derived matrices (e.g., collagen and alginate), acellular tissue matrices (e.g., acellular bladder submucosa and small intestinal submucosa (SIS)), and synthetic polymers. Naturally derived materials and acellular tissue matrices have the advantage of being biologically recognized, but their production needs to be closely controlled and monitored to maintain good and reproducible quality. Synthetic polymers, however, can be reproducibly manufactured in large quantities with good quality and standardized physical properties.

In many cases, a cell source is necessary to fully replace the organ's metabolic, exocrine, or endocrine functions. The source of donor tissue can be **xenogeneic** (from another species), **allogeneic** (same species, different individual), or **autologous** (same individual). Autologous cells are the preferred source as they avoid rejection from the host and preclude the use of immunosuppressive drugs. However, many patients with a specific organ disease no longer possess the specific healthy tissues or cells that should be used for culture expansion and subsequent transplantation. In these instances, donor cells or tissues are required to serve as an appropriate cell source. Another cell type with great potential applicable to many different organs could be the use of stem cells. It is now known that human embryonic stem cells (hESCs), for example, are capable of self-renewal, proliferating in an undifferentiated state while maintaining their pluripotent properties. We also know that we can differentiate these cells into almost any specialized human tissue cell type when providing the correct culture conditions. Researchers are

making progress in directing these and other stem cells toward specific cell lineages with the goal of regenerating healthy organs that can replace diseased ones without depending on the availability of donor cells.

Engineered tissues exceeding a volume of 3 mm^3 cannot survive adequately because of impaired nutrition and gas exchange. Vascularization of the regenerating cells is mandatory to engineer large complex tissues. Progress toward this goal is being achieved by incorporation of angiogenic growth factors into the engineered tissue, by seeding endothelial cells in combination with the other cell types (including stem cells) and by prevascularization prior to cell seeding. There is no doubt that the problem of tissue construct vascularization has to be solved before large entire solid organs can be regenerated (see [Chapter 14](#) vascularization, survival, and functionality of tissue-engineered constructs).

19.3 Urogenital tissue engineering

The urogenital system comprises the urinary tract and the internal and external genital apparatus. The urinary tract is subdivided into the upper tract (comprising the kidney and ureter) and the lower tract (comprising the bladder and urethra). In the female, the ovaries, the uterus, and the vagina belong to the internal genital apparatus. The external apparatus comprises the introitus, in the female the minor and major labia, and in the male the testicles and the penis, and, in particular, the corporal structures ([Fig. 19.1](#)).

19.3.1 Bladder

Intestinal and, less frequently, gastric segments are normally used as tissues for bladder replacement or repair. However, intestinal tissues are designed to absorb, and when they come into contact with the

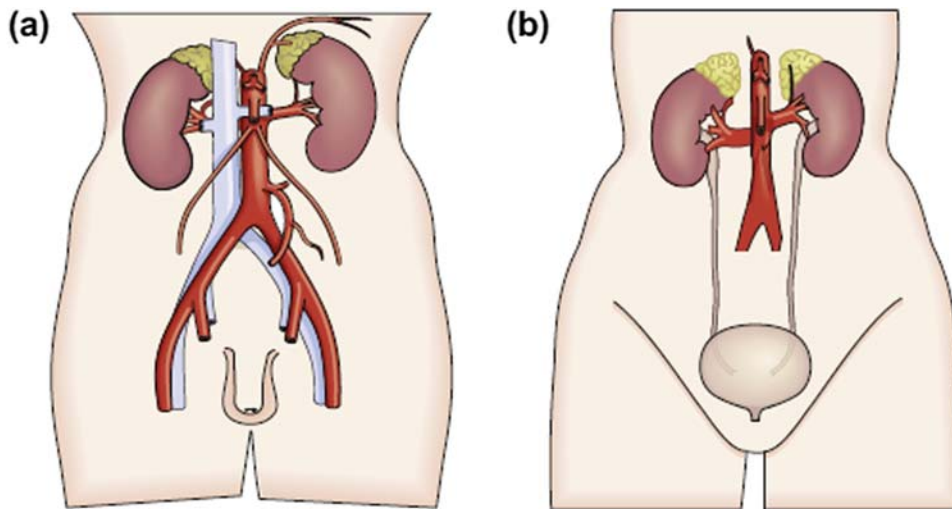


FIGURE 19.1

Schematic representation of the human urogenital system. (a) Vascular anatomy, (b) Upper urinary tract.

urinary tract, they also absorb toxic substances normally eliminated with the urine. Therefore, multiple complications may ensue, such as infection, metabolic disturbances, increased mucus production, stone formation, and even malignancies. The use of gastric tissue may induce metabolic disturbances or even perforation. Owing to the problems encountered with the use of gastrointestinal segments, numerous investigators have attempted alternative reconstructive procedures for bladder replacement or repair, such as the use of seromuscular grafts, matrices for tissue regeneration, and tissue engineering with cell transplantation.

In the bladder, the trigone is a smooth triangular region of the inner urinary bladder formed by the two ureteral orifices and the internal urethral orifice. Cell-seeded allogeneic acellular bladder matrices have been used for bladder augmentation in dogs. A group of experimental dogs underwent a trigone-sparing cystectomy and were randomly assigned to one of three groups. One group underwent closure of the trigone without a reconstructive procedure, another underwent reconstruction with a nonseeded bladder-shaped biodegradable scaffold, and the last underwent reconstruction using a bladder-shaped biodegradable scaffold that delivered seeded autologous urothelial cells and smooth muscle cells.¹²

The cystectomy-only and nonseeded controls maintained average capacities of 22% and 46% of preoperative values, respectively. However, in the cell-seeded tissue-engineered bladder replacements, an average bladder capacity of 95% of the original precystectomy volume was achieved. The compliance of the cell-seeded tissue-engineered bladders was also superior. Histologically, the nonseeded scaffold bladders presented a pattern of normal urothelial cells, however, with a thickened fibrotic submucosa and a thin layer of smooth muscle fibers. The retrieved tissue-engineered bladders showed a normal cellular organization, consisting of a trilayer of urothelium, submucosa, and smooth muscle. This technology was applied in seven patients with **myelomeningocele** with high-pressure, or poorly compliant bladders (State-of-the-Art Experiment). Researchers followed this study with a multicenter phase two clinical trial to evaluate bladder capacity, compliance, and safety in 10 pediatric patients with **neurogenic bladder** secondary to spina bifida.¹⁰ Unfortunately, no statistically significant improvement in bladder capacity or compliance was observed. The technology is now moving to additional human trials following the same parameters utilized during the initial clinical experience.

19.3.2 Urethra

In a rabbit model, urethral replacement was performed with tubularized collagen matrices either seeded with cells or without cells. The matrices seeded with autologous cells formed new tissue, which was histologically comparable to native urethra. However, the tubularized collagen matrices without cells led to limited tissue development, fibrosis, and stricture formation. It was determined through a series of studies that the maximum distance for normal cell regeneration across an acellular graft in vivo was 0.5 cm from the edge of normal tissue. Larger gaps would tend to fibrose and stricture.

These results were confirmed clinically in a series of patients with a history of failed **hypospadias** repair wherein the urethral defects were repaired with human bladder acellular collagen matrices, showing the first demonstration in humans that acellular grafts could be used for normal tissue regeneration inside the body.¹² The neourethral tissue was created by connecting the matrix to the urethral plate in an onlay fashion, replacing only the top half of the urethral defect, thus observing the 0.5 cm rule of normal tissue regeneration from any normal edge. The size of the created neourethra ranged from 5 to 15 cm.

After a 3-years follow-up, three of the four patients had a successful outcome in regard to cosmetic appearance and function. One patient who had a 15-cm neourethra created developed a subglandular fistula that required minor repair. Similar results were obtained in pediatric and adult patients with primary urethral stricture disease using the same collagen matrices.

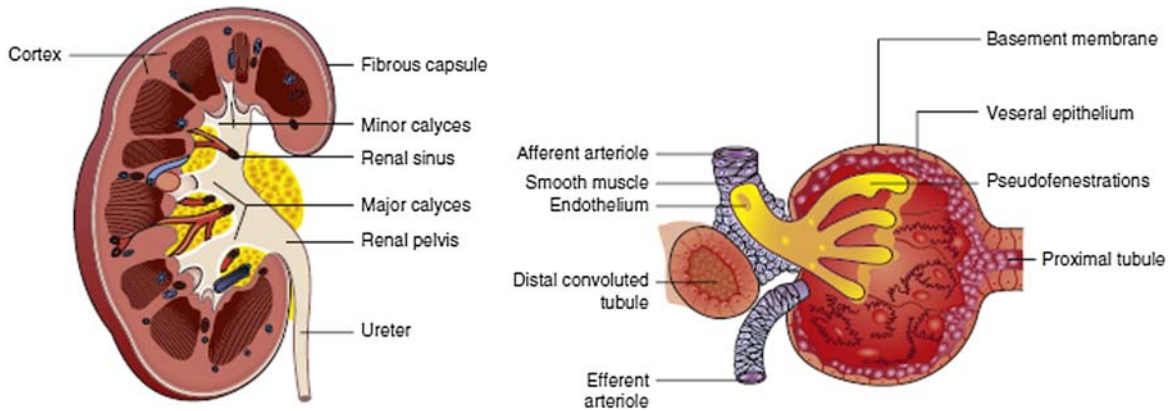
The ultimate goal was to provide fully engineered tissues to patients requiring complete substitution urethroplasties, such as those with long strictures or significant penile trauma. Poor success was reported after initial attempts in five patients with significant stricture secondary to lichen sclerosis. Keratinocytes and fibroblasts isolated from buccal mucosal biopsies were seeded on acellular dermal grafts used for complete substitution tubularized urethral replacement. Following this attempt, a study reported the successful implantation of bioengineered urethras into five boys suffering severe strictures. Open bladder biopsies harvested 1-cm² full-thickness tissues that were divided into urothelium and smooth muscle, and then cultured separately in the laboratory. These autologous tissues were then seeded onto the intraluminal and outer surfaces of a tubularized PGA acellular matrix for treatment of defects 4–6 cm in length. Of the patients, 100% demonstrated continence with normal flow rates and continence at a median follow-up of nearly 5 years. Further, cellularized implants did not exhibit severe fibrosis, inflammation, or stricture formation, and demonstrated histologically normal tissue on follow-up biopsies. Work in this area continues with a subsequent FDA-approved clinical trial.

19.3.3 Kidney

The kidney is a highly complex organ, and the gold standard for treatment of kidney failure resulting from end-stage renal disease (ESRD) is transplantation. However, transplants expose patients to the risk of developing infection, rejection, and on-going costs for medical care. The current demand for kidney transplantation is also substantially greater than that of organ supply; this puts transplantation out of reach for many patients who require it. The alternative is the commonly used dialysis to replace absent renal function, but this is associated with a relatively high morbidity and mortality, and only partially replaces healthy kidney function.

The kidney is a highly complex organ making it incredibly difficult to reconstruct by tissue engineering methods because of its complex structure and function (Fig. 19.2). Unlike hollow or viscous organs, the kidney's solid architecture presents the unique challenge of engineering several structural and functional capabilities into a single organ. The challenge is to include the normal kidney's one million highly specialized nephrons working in concert to drain into a single renal pelvis. Two approaches have been used to address the need for **bioartificial** kidney: (1) replacement of isolated kidney function parameters with the use of **extracorporeal** cellular units and (2) replacement of total renal function by tissue-engineered bioartificial structures.

To form isolated cellular units, investigators use *ex vivo* cellular systems, in an attempt to assess the viability and physiologic functionality of a cell-seeded device to replace the filtration, transport, and metabolic and endocrinologic functions of the kidney. One example of this is the formation of kidney **organoids**. One group has utilized developmental mechanisms that regulate differentiation of kidney progenitors to form the kidney structures. The small organ-like structures contain nephrons and collecting duct networks with a pseudorenal interstitium with endothelial cells.¹³ These kidney organoids represent powerful models for future applications, including disease modeling and nephrotoxicity

**FIGURE 19.2**

The complex renal structure. A cross-section of the whole kidney (*left*) and the glomerular apparatus (*right*).

screening, and could even be used as building blocks for whole organ kidney engineering. Alternatively, some groups have attempted to direct structure formation through the use of enabling technologies like bioprinting. In one model, a 3D human renal proximal tubule was printed into an extracellular matrix (ECM) and formed into a microfluidic chip. The engineered tubule is lined with confluent epithelium and endothelium, with active reabsorption via the tubular–vascular exchange.⁸ As a next step, our team has utilized a photo-crosslinkable kidney ECM-derived **bioink** (KdECMMA) to provide a kidney-specific microenvironment for renal tissue bioprinting. To do so, porcine whole kidneys are decellularized through a perfusion method, dissolved in an acid solution, and chemically modified by methacrylation. Bioprinted renal constructs printed with human kidney cells in KdECMMA bioink exhibit the structural and functional characteristics of the native renal tissue. This multifactorial approach to kidney bioprinting improves end organ function.¹

Alternatively, Bertram et al. have shown that a population of selected renal cells stabilize disease progression in a mass reduction model of chronic kidney disease. First the group developed methods that yield distinct subpopulations of primary kidney cells that are compatible with process development and scale-up. These methods were translated to rodent, large mammal, and human kidneys, and then to rodent and human tissues with advanced chronic kidney disease. Injection of **syngeneic** renal cells into the renal cortex of rats with progressive nephropathy secondary to diabetes, obesity, dyslipidemia, and hypertension elicited a regenerative response that significantly improved survival and stabilized disease progression to renal structure and function. The researchers found multiple nephron structures and functions including glomerular filtration, tubular protein handling, electrolyte balance, and the ability to concentrate urine. Improvements to blood pressure, including reduced levels of circulating renin, were also observed. These functional improvements following renal cell treatment were accompanied by significant reductions in glomerular sclerosis, tubular degeneration, and interstitial inflammation and fibrosis. Collectively, these data support the utility of a novel renal cell-based approach for slowing renal disease progression associated with diabetic nephropathy in the setting of metabolic syndrome, one of the most common causes of ESRD (Bertram, 2012). As a first in human study, the group used laparoscopically assisted

implantation of selected renal cells in patients with chronic kidney disease. Unfortunately, kidney volume, split function, and glomerular filtration rate did not change during 12 months of follow-up. An extended 24-month follow-up in five of the patients showed a decline in estimated glomerular filtration rate (cystatin C).¹⁸ Our group has also looked at the use of cell therapy to augment and restore renal function by utilizing amniotic fluid stem cells (AFCs). Amniotic fluid stem cells (AFSCs) share advantages of both embryonic and adult stem cells, such as pluripotent activity, remarkable plasticity, and immunomodulatory effects, which may allow their future therapeutic use as an “off-the-shelf” cell source. Our findings demonstrate that administration of human-derived AFSC facilitates functional and structural improvement in a rat model of chronic kidney disease and suggests that cell therapy with AFSC has potential as a therapeutic strategy to recover renal function in patients with CKD.

19.4 Reproductive organs

19.4.1 Uterus

The uterus is the largest and major organ of the female reproductive system. It is a hollow, muscular tissue located in the female pelvis situated between the bladder and rectum and is hormonally responsive. The main function of the uterus is to nourish the developing fetus prior to birth. Anatomically, on one end is the cervix which opens into the vagina, the other is connected to the fallopian tubes. The reproductive function of the uterus is to accept a fertilized ovum which passes through the uterotubal junction from the fallopian tube. The fertilized ovum is then implanted into the endometrium and blood vessels develop to provide nutrients for this purpose. In rodent models, small uterine defects regenerated when repaired with a variety of biomaterials alone, without the use of cells, such as nondegradable synthetic polymer scaffolds, biodegradable synthetic polymer scaffolds, naturally derived scaffolds, and even scar tissue. However, attempts at regenerating larger uterine defects using these strategies have failed. Moreover, rodent organs, including the uterus, have an inherent regenerative capacity. Thus, results obtained in rodent models may not translate to larger animal models and humans.

A recent study used biodegradable polymer scaffolds seeded with autologous cells to restore uterine structure and function in rabbits. Rabbits underwent a subtotal uterine excision and were reconstructed either with autologous cell-seeded constructs, with nonseeded scaffolds, or by suturing. At 6 months postimplantation, only the cell-seeded engineered uteri developed native tissue-like structures, including organized luminal/glandular epithelium, stroma, vascularized mucosa, and two-layered myometrium. The bioengineered construct supported stretch-induced tissue expansion (more than 10 times its weight) and remodeling that occurs during pregnancy to accommodate the growing fetus, placenta, and amniotic fluid, and supported formation of viable offspring with body weights similar to those of the normal controls, which suggests normal placental function. Only rabbits with cell-seeded constructs had normal pregnancies within the reconstructed segment of the uterus and supported fetal development to term and live birth. With further development, this approach may provide a regenerative medicine solution to uterine factor infertility.¹¹

19.4.2 Vagina

The vagina is a fibromuscular tubular track with the primary function of intercourse as well as childbirth. Similar to other parts of the female reproductive organs, several pathological conditions,

including congenital malformations like Mayer–Rokitansky–Kuster–Hauser (MRKH) syndrome, cloaca or thick transverse vaginal septum, obstetric trauma, and malignancy, can adversely affect normal vaginal development or anatomy. Vaginal reconstruction has traditionally been challenging due to the paucity of available native tissue. The feasibility of engineering vaginal tissue *in vivo* has been investigated. Vaginal epithelial and smooth muscle cells of female rabbits were harvested, grown, and expanded in culture. These cells were seeded onto biodegradable polymer scaffolds, and the cell-seeded constructs were then implanted into nude mice for up to 6 weeks. Immunocytochemical, histological, and Western blot analyses confirmed the presence of vaginal tissue phenotypes. Electrical field stimulation studies in the tissue-engineered constructs showed similar functional properties to those of normal vaginal tissue. When these constructs were used for autologous total vaginal replacement, patent vaginal structures were noted in the tissue-engineered specimens, while the noncell-seeded structures were noted to be **stenotic**. The first case of creation of a human vagina using autologous *in vitro* cultured vaginal tissue has also been reported.¹⁴ The patient had **MRKH syndrome (MRKHS)** and tissue was grown from a 1 cm² biopsy of vaginal vestibular mucosa. Autologous reconstructed vaginal tissue reached 314 cm² in 2 weeks. A procedure was then performed to create a vaginal space to allow inlay of cultured tissue. Clinical exam demonstrated a vaginal vestibular mucosa with normal length and depth with normal vaginal tissue on biopsy [67] (Fig. 19.3).

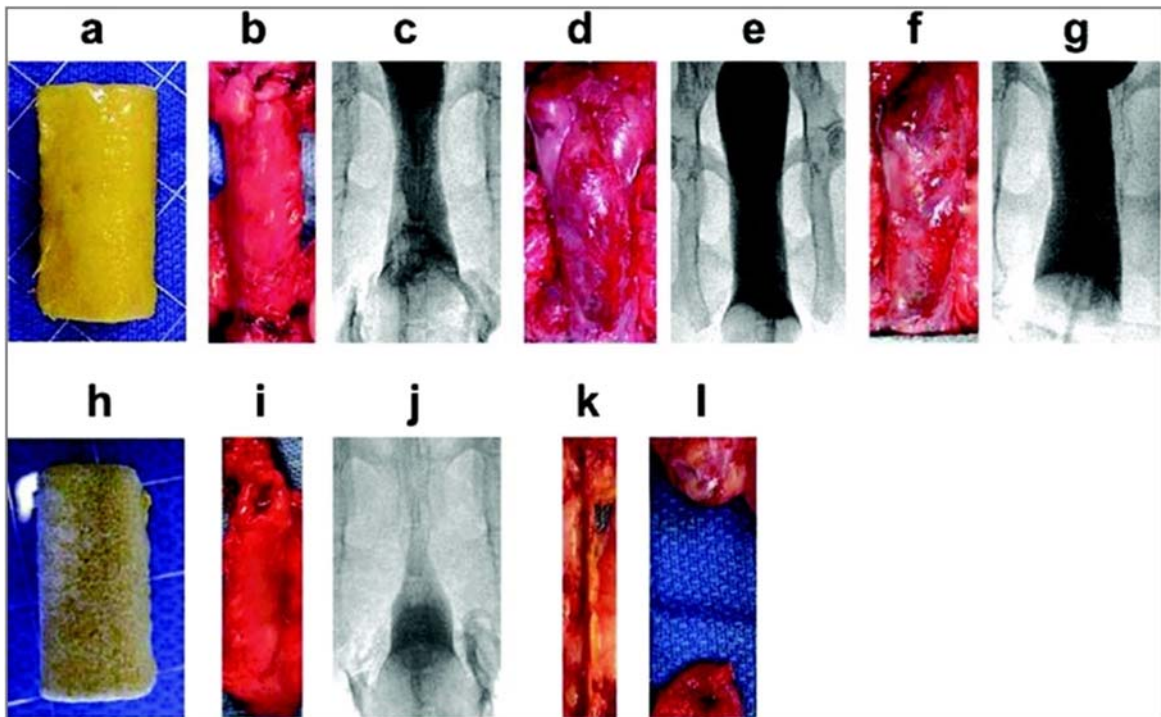


FIGURE 19.3

Appearance of tissue-engineered neovaginas. (a) Tubular polymer scaffold after cell seeding and 1 week *in vitro* culture, before implantation *in vivo*. (b, d, f) Gross appearance and (c, e, g) vaginography of cell-seeded constructs 1, 3, and 6 months, postimplantation, respectively. (h) Unseeded control scaffold before implantation. (i, k, l) Gross appearance of unseeded construct at 1-, 3-, and 6 months postimplantation. (j) Vaginography of unseeded graft at 1 month. *From De Filippo RE, et al. Transplantation 2008 July 27;86(2):208.*

In addition, a study using four patients who suffered from congenital vaginal aplasia due to MRKHS over a 3-year period received vaginal organs engineered from their own cells and implanted back into the patient. Finding from this study showed normal structural and functional variables with follow-up of up to 8 years¹⁵ (Fig. 19.4). These technologies could be useful in patients requiring vaginal reconstruction surgeries.

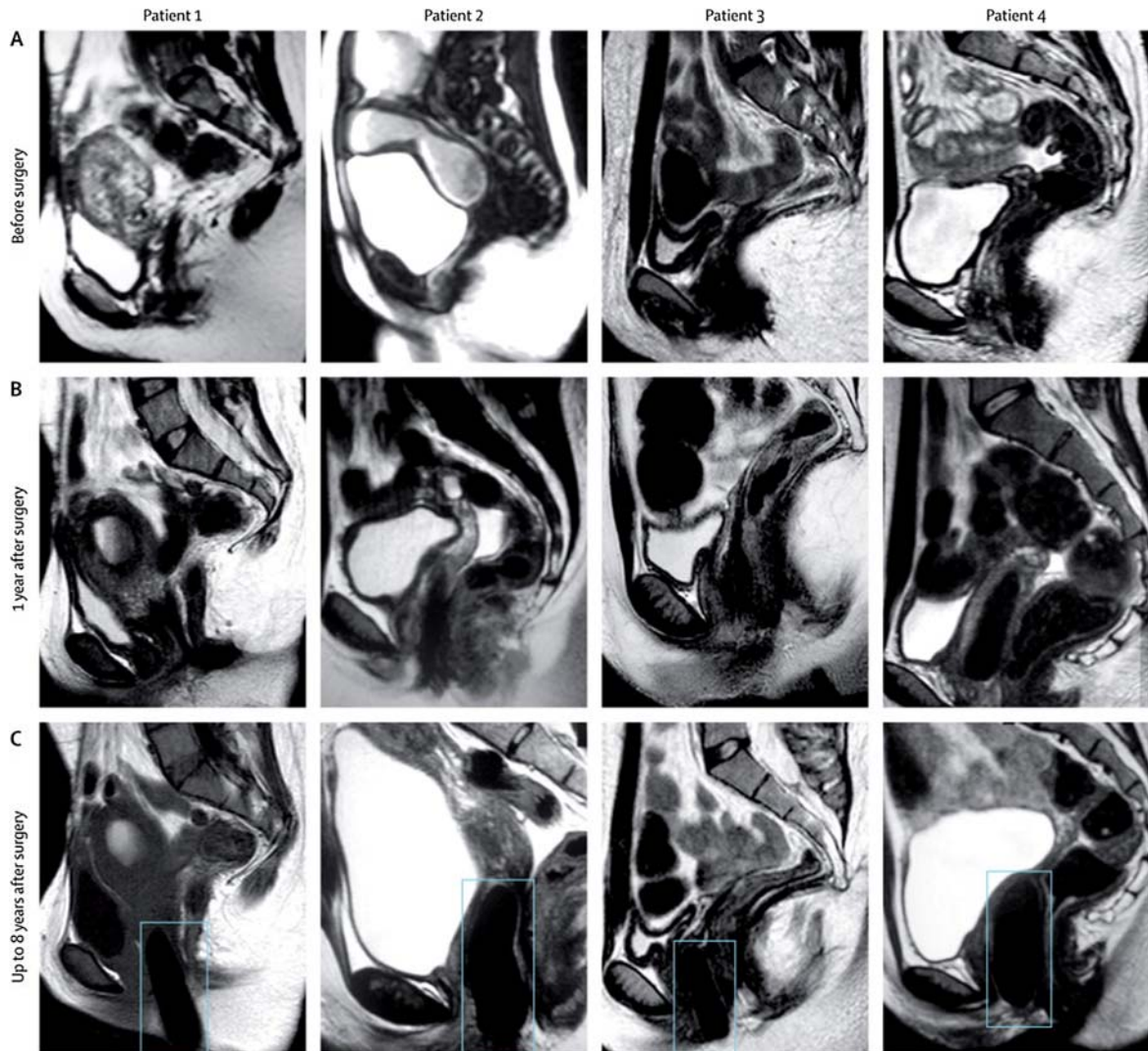


FIGURE 19.4

Preoperative and postoperative MRI images. (a) Preoperative MRI images show absence of vaginal organs. (b) MRIs 1 year after surgery show engineered vaginal organs. (c) Latest MRI images up to 8 years after surgery (boxes within the MRIs show engineered vaginal organs). From Raya-Rivera AM, et al. *Lancet* 2014 July 26;384(9940):329, July 26, 2014.

19.4.3 Ovaries

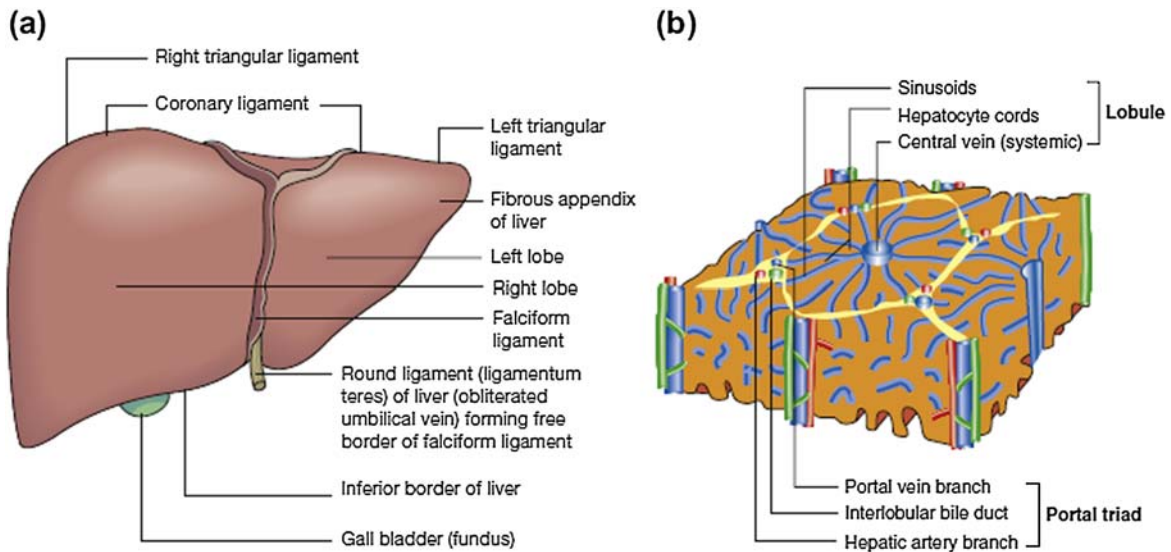
The ovaries are the ovum-producing organs of the internal female reproductive system. They are typically found in pairs as part of the vertebrate female reproductive system. The fundamental role of the ovary is to produce oocytes capable of fertilization as well as to secrete the hormones estrogen and progesterone that will prepare the uterus for successful implantation. Ovarian sex steroids also play a major role in breast development, bone health, and sexual function in the female. A number of pathological conditions such as premature ovarian failure and cancer chemotherapy may severely compromise reproductive function. In addition, with the decline of sex steroid hormones around natural menopause, significant pathology, such as vasomotor symptoms, genital atrophy, and bone loss, ensues. In the attempt to restore a functional ovary, several approaches have been employed at the experimental level. Some of the significant approaches include regenerating ovarian tissues from stem cells transplanting ovarian tissues and use of tissue engineering tools.

It was previously considered that germ cell renewal (formation of oocyte and follicle) is restricted to fetal life. A group of stem cells (germ stem cells, GSCs) is present in the ovarian surface epithelium that stain positive for a germ cell–specific marker, the mouse vasa homologue, using **BrdU (5-bromo-2'-deoxyuridine) staining**. They seem to maintain the oocyte and follicle production in the postnatal ovary, but a debate on the existence of these stem cells in adult mammalian ovaries and their potential significance is ongoing. The differentiating cells were reported to have various limitations including their heterogeneity, which makes the *in vitro* oogenesis from these stem cells challenging.⁷ More than a decade later, stem cells are still being studied in their capacity for regenerative purposes in the ovaries.

Recently, new *in vitro* culture methods involving tissue-engineered matrices have been developed to study the maturation of ovarian follicles. The 2D culture systems support the production of immature mouse follicles or granulosa cell–oocyte complexes where the granulosa cell is attached to the culture substrate and migrates away from the oocyte.¹⁶ On the other hand, for the purpose of cell-based hormone replacement therapy, our group has developed an engineered multilayer ovarian tissue model that secretes sex steroids and peptide hormones in response to gonadotropins *in vitro*. Theca and granulosa cells placed in different layers of multilayer alginate microcapsules that mimic the architecture of native follicles and their endocrine functions were found to be more potent than two other possible combinations. Similarly, encapsulated endocrine cells implanted into the peritoneum of rodents have been reported to deliver hormones in ovariectomized animals.

19.5 Liver tissue engineering

The liver is a very complex organ that has the ability to regrow and regenerate (Fig. 19.5). However, liver failure can still occur due to many different causes, including acquired liver disease, inborn errors of metabolism, or drug toxicity. Currently, when a liver fails, the lost function can only be replaced fully by liver transplantation. The options include transplantation of the whole liver or parts of the liver from cadaveric donors, portions of the liver from living donors, or xenotransplantation, typically porcine. Unfortunately, there are far too few livers available for the need worldwide, with approximately five

**FIGURE 19.5**

Liver structure. (a) Surfaces and bed of liver. (b) Hepatic architecture.

times as many recipients as donors, and xenotransplantation is limited by immune and infectious complications.

The simplest method of liver failure treatment using hepatocytes is cell transplantation. The first liver cell transplantation was described in the 1970s, with the successful transplant of viable rat hepatocytes into the spleen in an animal study. This and other studies taught us valuable lessons about the importance of the microenvironment to cell survival, as the cells required conditions resembling those of the liver, including a matrix to grow on and a venous blood supply similar physiologically to the hepatic sinusoid to grow and function normally. They also demonstrated the role of nonhepatocyte cells in hepatocyte survival, as coculture with nonparenchymal cells was often required to ensure cell viability. Unfortunately, clinical experience shows that many patients treated with hepatocytes for acute liver failure die of sepsis and most trials investigating the treatment of fulminate liver failure with hepatocyte transplantation have given equivocal results.

For cells to be useful in transplantation, they must have a high proliferative capacity without loss of function, and they must be economically feasible. There are several groups of possible cell sources for liver replacement. These include primary hepatocytes, immortalized cell lines, stem cells, and trans-differentiated cells. Primary hepatocytes are the most desirable and commonly used cells. Unfortunately, hepatocyte function is closely tied to the orientation, location, and surrounding environment of the cells. These environmental characteristics are lost in culture, so these cells rapidly and irreversibly lose their function. One method to overcome these challenges is to use liver cell aggregates.

Human hepatocytes would be the best choice for cell transplantation, but just as liver donors are limited, so are human hepatocytes. Fetal or neonatal hepatocytes may also provide a source of cells.

Another source of hepatocytes is stem cells. Stem cells have an unlimited replication potential and can differentiate into various different cell types. Potential sources of stem cells for hepatocyte generation include induced pluripotent stem cells, adult liver progenitor cells, and transdifferentiated nonhepatic cells.

Another strategy of liver replacement in the setting of liver failure is bioartificial liver (BAL) replacement. As an evolution of the artificial liver devices, BAL constructs were developed to harness the function of hepatocytes in an extracorporeal system. BAL devices have been under development for >40 years and usually consist of nearly 15 billion isolated hepatocytes with a membrane through which the plasma is circulated. There are already several examples of BALs currently undergoing clinical trials, which include ELAD, HepatAssist, BLSS, AMC-BAL, MELS, RFB, and HBAL/TECA-HALSS. Unfortunately, only two clinical trials that explored the effectiveness of BALs have been reported, and it is clear that many challenges remain before widespread dissemination for clinical use can be achieved.

The creation of a complete, implantable bioengineered liver remains the ultimate goal of tissue engineering for liver replacement. Many strategies have been explored as a means to achieve this goal, such as the injection of hepatocytes into vascular beds or the use of implantable seeded scaffolds. One of the main limitations of bioengineering large organs such as the liver is vascularization to provide cells embedded in such constructs with adequate nutrient supply ensuring a proper physiological response. Prior to transplantation, researchers must have a fully functional bioengineered liver, as it must immediately perform all hepatic functions.

Animal studies have shown that the liver architecture remains viable after stripping and can be repopulated with hepatocytes. As early as 2011, researchers were able to generate a fully vascularized liver organoid after decellularization of livers across multiple species.³ After rescue with human fetal liver and endothelial cells, these *in vitro* constructs were able to withstand fluid flow through the vascular network and generated significantly higher concentrations of urea and albumin than equivalent hepatic cells grown in culture dishes. Taken with other studies in this field, these technologies are directing the next phase of research in regenerating a viable liver construct for therapeutic use in humans by overcoming the challenges of oxygen and nutrient delivery, waste removal, and immunologic rejection. Researchers still must overcome challenges associated with establishing an ideal cell source, maintaining *in vivo*-like oxygen conditions, achieving bile secretory function, increasing convenience for the patient, and establishing efficacy of the devices overall.

Other groups have demonstrated that hESCs or human-induced pluripotent stem cells can be used to generate hepatocyte-like cells. In one study, a bioprinting model used a valve-based inkjet printer to fabricate mini livers, which demonstrated both nuclear factor 4 alpha and albumin secretion. The printed cellular alginate hydrogel matrix reached peak albumin secretion after differentiation for 3 weeks, the longest *in vitro* culture published at the time. It also demonstrated that valve-based printing did not damage human pluripotent stem cells and helped maintain their pluripotency, followed by directed differentiation into specific lineages of patient-specific hepatic cells. More recently, primary rat hepatocytes, HUVECs, and human lung fibroblasts were printed with an extrusion bioprinter. In the design, polycaprolactone formed a framework, while collagen bioink containing the cells was infused into the support structure. The resulting 3D coculture environment induced capillary network formation, which facilitated hepatic cell growth. The hepatocytes demonstrated the

ability to secrete albumin and synthesize urea, suggesting that the heterotypic interaction among hepatocytes and the newly formed vascular network increased the survival and function of hepatocytes in collagen hydrogel. While these findings are promising, the complexity of the liver microvascular network and anastomosis with the bile duct, portal vein, and hepatic artery have yet to be realized. These elements will be essential to the scale-up of current tissue-engineered liver models into clinically relevant liver tissue.

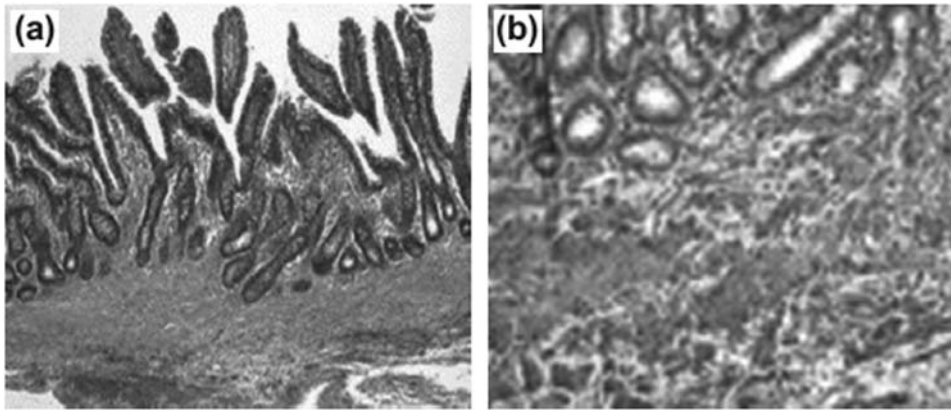
19.6 Gastrointestinal tissue engineering

The current best therapy for replacing diseased or absent intestine is organ transplantation, aimed at restoring intestinal function for adequate nutrient absorption, fluid and electrolyte transport, and restoration of **metabolic homeostasis**. This involves an extensive operative procedure with its own morbidity and mortality, along with the well-described side effects of chronic **systemic** immunosuppression. There are also the concerns of inadequate numbers of organ donors and rejection of the transplanted tissue. An excellent alternative to current therapies would be to regenerate intestine using tissue engineering techniques that have been successfully applied to other organs.

Early attempts at tissue engineering bowel segments involved patch repairs of intestinal defects. As far back as the 1960s, jejunal serosa has been tested experimentally to repair duodenal defects in dogs. These patches were covered by duodenal epithelium in 2 months. Expanding their work, they used a model of “short bowel syndrome” in pigs to show that the jejunal patch was able to induce increased weight gain. The studies concluded that these patches were all able to support the growth of functional neomucosa.

19.6.1 Natural biomaterials for intestinal repair

Acellular and natural scaffolds have recently been found to be highly effective for bowel repair. An example of a widely used acellular biomaterial is small intestinal submucosa (SIS). SIS is primarily composed of ECM material, following the removal of the mucosa, external muscle layers, and other cells from the small intestine of pigs. The naturally occurring ECM is rich in components that support angiogenesis (i.e., fibronectin; glycosaminoglycans; collagen types I, III, IV, and V; bFGF; and vascular endothelial growth factor). SIS has also been shown to be nonimmunogenic. SIS has been extensively investigated as a scaffold in numerous tissue engineering applications and is now processed in a standardized fashion in mass quantities; this makes it readily available for tissue engineering studies. SIS has been used to cover defects in the esophagus and small bowel. In these studies, the SIS patch allowed the mucosa to grow over the scaffold, and the histology of the patch was similar to the native intestine. In another study, tubular noncell containing SIS grafts from Sprague–Dawley rat donors was used to create an ileostomy (Fig. 19.6). These grafts were initially covered by inflammatory cells, but by 3 months, they were completely covered by mucosa. Also, the outsides of the tubular grafts were covered with bundles of smooth muscle-like cells and fibrovascular tissue. These studies demonstrated that SIS could develop structural features of the normal intestine and may provide an off-the-shelf option for replacement patches for intestinal tissue.

**FIGURE 19.6**

Histologic photomicrographs of SISs-regenerated rat small bowel tissue at 12 weeks. (a) Well-organized mucosal epithelial layer with smooth muscle cells regeneration. (b) Immunohistochemically stained intestinal tissue suggesting bundles of well-formed smooth muscle fibers with less regularity.

19.6.2 Combining biomaterials with cells for intestinal repair

The key components for intestinal tissue engineering include a cell source, and an appropriate biocompatible and biodegradable scaffold. Early work in intestinal tissue engineering used just such a strategy. Cells were obtained from an intestinal organoid unit; a scaffold of sheets of collagen-coated, microporous, nonwoven PGA was formed into a tubular shape, and these tubes once seeded with cells were implanted into the **omenta** of animals.¹⁹ The authors harvested cells from intestinal organoids, seeded these on the constructs, and after subsequent implantation, they found it decreased morbidity associated with bowel resections in rats. However, such constructs have not demonstrated physiologic functionality; this shows again that a proper combination of cells and biomaterials is critical for a good outcome. Although these implants have now demonstrated basic metabolic function, one important aspect is still missing, namely, integration with the enteric nervous system that is crucial for a normal bowel motility.

There are however indications that nerve innervation, or at least the stimulation of nerve ingrowth, is possible by using the right cell type. Notable success has been achieved with the implantation and subsequent neovascularization of bioengineered internal anal sphincters in murine models. Implanted constructs have demonstrated maintenance of basal tone and sphincteric relaxation similar to native sphincter. One group described their method to bioengineer functionally innervated gastrointestinal smooth muscle constructs using neuronal progenitor cells and smooth muscle cells that were isolated and cultured from intestinal tissues of adult human donors.⁵ This breakthrough in bioengineering functional gastrointestinal tissue *in vitro* provides not only a useful construct for the examination of the disease processes of the bowel but also a possible “off-the-shelf” source of intestinal tissues with physiological and metabolic functions for clinical use.

19.7 Pancreas tissue engineering

Diabetes mellitus is a common disease, which affects 4% of the world, and causes significant morbidity and mortality through its effects on vascular/microvascular disease. Diabetes is due to systemic hyperglycemia, resulting from inadequate insulin production by the pancreas and/or resistance to insulin.²⁰

The insulin-producing β cells can be found in the pancreatic islets, or islets of Langerhans. The pancreas is composed of acinar cells, ducts, and islets of endocrine cells. Pancreatic cells develop from endodermal progenitor cells. The islets contain a specific set of endocrine cells comprising the β cells, α cells, which produce glucagon, δ cells, which produce somatostatin, ϵ cells, which produce ghrelin, and PP cells, which produce pancreatic polypeptide. These endocrine cells all work in unison and stimulate, or inhibit, each other through paracrine effects. Although the islets only take up a very small portion (1%–2%) of the total pancreas volume, they receive 10%–15% of the blood flow that increases when blood glucose levels are elevated. Each islet therefore is densely vascularized, and islets cells are never further away than 100 μm from a blood vessel to provide them with sufficient amounts of nutrients and oxygen and to efficiently release insulin into the bloodstream (Fig. 19.7).

There are two main types of diabetes, types I and II, and they are caused by islet β cell dysfunction, with an absolute or relative decrease in insulin production. Type I diabetes results from the autoimmune destruction of β islet cells. Type II diabetes is characterized by systemic insulin resistance, an inability of the β cell to rapidly release sufficient insulin in response to oral nutrient stimulation, and to impaired

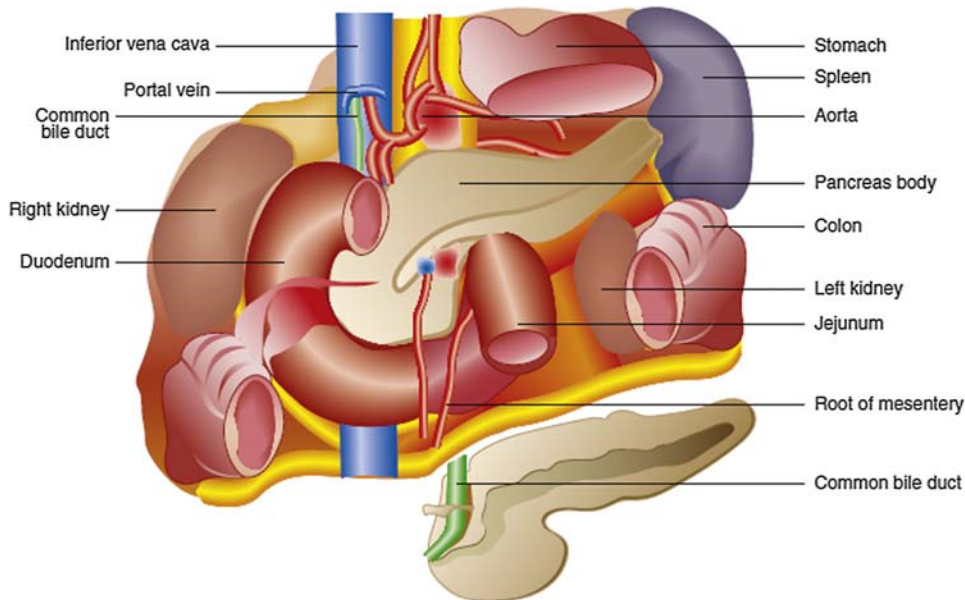


FIGURE 19.7

The pancreas anatomy and surrounding organs.

glucose tolerance. Also, β cells may not be able to convert proinsulin, which is the precursor in the cell for forming insulin.

The most effective therapy for replacing lost β cell function, since the discovery of insulin by Banting and Best in 1921, has been insulin replacement therapy.⁴ The ability to monitor blood glucose levels and provide patients with insulin on a daily basis multiple times a day works for many patients today. However, it is still very difficult to match insulin levels appropriately to the blood glucose concentration in real time. Scientists therefore continue to search for new therapies to improve the treatment of diabetes mellitus, such as β cell replacement, which seeks to replace the deficient endogenous insulin production especially for type I diabetes patients.

Engineering of new β cells involves two approaches: gene therapy and cell replacement strategies. Engineered β cells need to be able to modulate basal insulin release for normal events such as exercise or infection and respond to continuous changing blood glucose levels. Keep in mind that the islets of Langerhans are innervated by the sympathetic, parasympathetic, and sensory nerve fibers, so that engineered β cells need to respond to neural input. Over life, there are changing demands on insulin release that are the result of changes in insulin sensitivity, such as in puberty, pregnancy, and old age. There is quite a challenging list of criteria an in vitro engineered β cell should obey. The cells should be able to increase their replicative capacity and **involute** when no longer needed. Cells should also produce insulin and appropriately process proinsulin to insulin to function properly, which is highly dependent on adequate oxygen supply. Preferably, there has to be a way to recreate the influence of gastrointestinal-derived factors in the modulation of insulin release. β cells normally release the hormone insulin in a pulsatile fashion, so the newly generated β cells should mimic this behavior.

A major concern for scientists in β cell engineering is the recurrence of disease. Type I diabetes has an immunological cause, so it may recur with in vitro differentiated cells, if they express the antigens leading to an autoimmune reaction. The causes of type II diabetes are not as clear, so recurrence could be a possibility here as well. Therefore, in vitro generated β cells may require immunosuppression, and these agents (specifically glucocorticoids) can cause insulin resistance, suppress insulin release, and prevent insulin processing. They also have other more severe risks, such as malignancy. Also, proinsulin may pose a cardiovascular risk, so proper processing of the hormones is essential.

19.7.1 Creating new β cells for cell therapy in type I diabetes

Based on the aforementioned challenging checklist, the cells developed must have certain physiologic functions, such as insulin gene expression, appropriate posttranslational modification of insulin, glucose-sensitive insulin production, and, ideally, be available in an unlimited supply to be used as cell therapy for diabetes mellitus. All these characteristics are typical of β cells, but this does not mean that one needs β cells to replace them; just cells that act like them would suffice. For example, modification of genes in cells that have similar origins to pancreatic cells may produce β cell-like qualities, such as the transfer of the Pdx-1 gene into mouse liver cells. These modified liver cells can activate gene expression of insulin 1 and 2 and the prohormone convertase, but unfortunately are not sensitive to glucose in the environment. It has also been demonstrated that ductal cells also, which comprise the ducts in the pancreas through which digestive enzymes are secreted, can be modified to produce insulin if modified with gene transfection.

But what about a stem cell approach for replacement of exogenous insulin therapy? The goal of stem cell therapy, as with all studies of multipotent cells in regenerative medicine, is the directed differentiation of these cells and the introduction of specific characteristics to these cells via genetic manipulation. There are several different choices of cells with which to work in this field of study, such as ES cells and adult stem cells. However, it is still somewhat unclear if there is a pool of adult stem cells in the pancreas and whether the duct cells can act as islet progenitors, or even if β cells can renew themselves.

Is there a role for stem cells in diabetes cell therapy? Perhaps, there has been some recent success in exploring strategies using ES cells and other stem cell types that can now be fully differentiated into cells with functional characteristics of native β cells. For example, some researchers have been using precursors, such as adipose-derived stem cells, capable of glucose regulation in mice. Yet, it has been considerably challenging to achieve a full β cell phenotype in vitro, and multiple studies have suggested the transplant site milieu to be an important factor necessary for terminal differentiation, including the effect of in situ signals.

Similar to tissue engineering of other organ systems, there is interest in using scaffolds and/or tissue constructs to regenerate a 3D organ that mimics the natural environment of the native endocrine pancreas compartment. It may even prove necessary for efficient terminal differentiation. Multiple groups have explored this option in rodents with some success, including the constitution of viable vascular networks and insulin secretion for several weeks. Although relatively new in the field of tissue engineering, there is clearly an increasing trend toward using regenerative medicine- and bioengineering-based strategies for the creation of a bioartificial organ that mimics the endocrine function of the native pancreas.

An additional component of pancreatic cell printing is the ECM microenvironment. Investigators have studied the effect of matrix-integrins on beta-cell function and viability following isolation. One group found that immediately after islets isolation, the peri-insular basement membrane was absent, and over 6 days, they saw a decline in islet attachment to the collagen matrix. Most recently, the decellularization process was used to produce ECM isolated from the pancreas to provide a tissue-specific microenvironment for islet cells printing. This process yielded in vitro culture of human islets with greater than 60% viability, with an associated increase in insulin secretion by islets printed in the ECM bioink. This process was further enhanced by the addition of endothelial cells and a layered, printed, 3D construct. The construct demonstrated advanced pancreatic functions including regulation of insulin secretion and maturation of insulin-producing cells from stem cell progenitors.

To address the need for viable β cells, Melton et al. developed a scalable differentiation protocol to generate millions of glucose-responsive β cells from hPSC. The stem cell-derived β cells (SC- β) were found to be phenotypically and functionally similar to native β cells. Specifically, the SC- β cells expressed markers found in mature β cells fluxed calcium in response to glucose, packaged insulin into secretory granules, and secreted insulin. Furthermore, the cells secreted human insulin into the serum in a glucose-responsive manner and ameliorated hyperglycemia when implanted into diabetic mice. Next, human SC- β cells were used for long-term glycemic correction, in a diabetic, immunocompetent animal. The SC- β cells were encapsulated with alginate derivatives and implanted into the intraperitoneal space of streptozotocin-treated mice. Glycemic correction occurred following implantation

without immunosuppression until removal at day 174 postimplantation. The grafts produced human C-peptide and, *in vivo*, glucose responsiveness was controlled to confirm the function of the implanted cells. Finally, implants removed after 174 days *in vivo* contained viable insulin-producing cells. Most recently, they reported an approach to generate SC- β cells from type 1 diabetic patients. The produced cells responded to different forms of β -cell stress *in vitro* and maintained normal β -cell function. They concluded that no major differences were present in SC- β cells derived from type 1 diabetic patients compared with SC- β cells derived from nondiabetic patients, suggesting that SC- β cells derived from type 1 diabetic patients could be used to treat the patient's diabetes in an autologous manner. By combining functional engineered pancreatic units such as insulin-producing organoids, encapsulated β -cells, and differentiated stem cells with bioprinting technologies that enable large-scale deposition and organization of these building blocks, there is potential for pancreatic engineering and replacement to be realized.

19.8 Lung tissue engineering

As in other fields currently being investigated by tissue engineers, organ transplantation is the preferred treatment and standard of care for organ failure for the lung. The lungs might seem to comprise a relatively simple anatomy, but in fact contain an elaborate network of branching bronchi, and ultimately end in very small alveoli that are covered by a thin layer of squamous epithelium that permits diffusion of oxygen into the dense microvascular network lining the epithelium on the inside. The trachea is lined by rings of cartilage to support the tubular structure that otherwise would collapse on itself (Fig. 19.8).

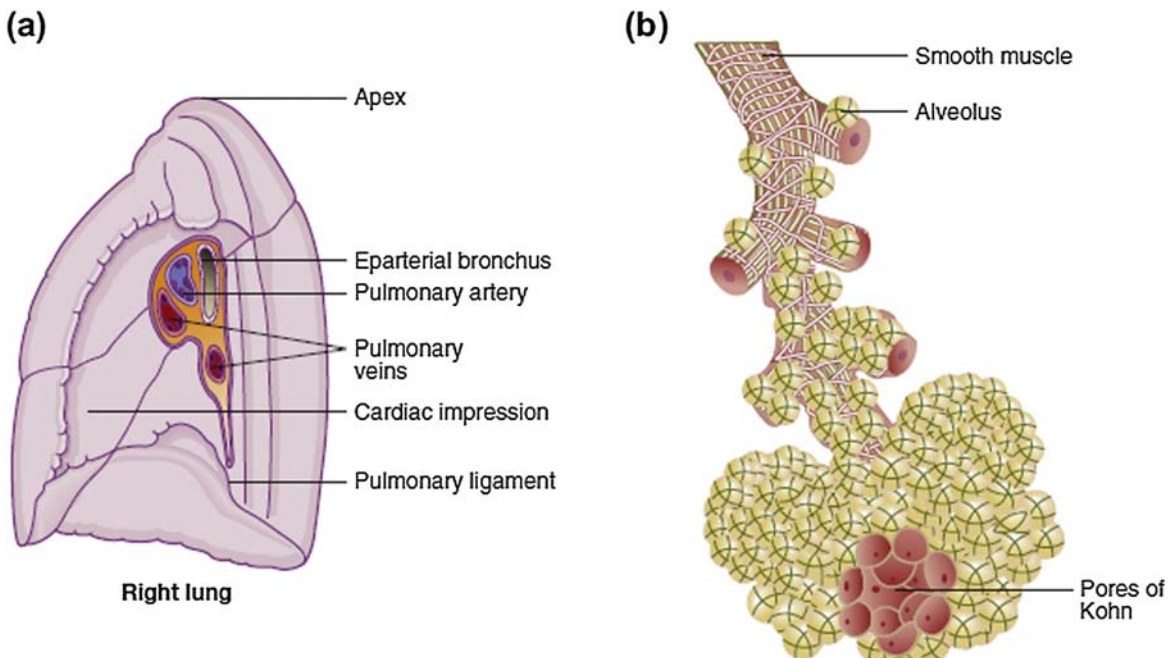


FIGURE 19.8

Anatomy of the lung. (a) A sagittal view of a right lung with its major blood vessels within the lung's hilum. (b) Bronchiole and alveoli where gas exchange occurs.

The demand for human organs for transplantation is large and growing, and the supply of donor lungs (as with most transplanted organs) does not meet the demand. Developing technologies for lung replacement include stem cell biology, therapeutic cloning, tissue and organ bioengineering, and xenotransplantation. Studies have demonstrated that ES cells can be induced to differentiate in vitro into pulmonary epithelial cells and type II pneumocytes, using a specific medium designed for the maintenance of lung epithelial cells in culture. The final goal of all this research is the ability to regenerate portions of the lung for replacement of damaged tissue. Clinically, the experience of this scale of bioengineering, while limited, is progressing.

Bioprinting has been identified as a technology capable of revolutionizing lung tissue engineering by allowing for precise engineering of thin tissue interfaces. In a recent study, Miller et al. generated biologically compatible complex multivascular networks with functional intravascular topologies.⁶ This was done using photopolymerizable hydrogels using a technique known as **SLATE printing**. Using this technology, the research created interwoven vascular networks with space-filling topologies and demonstrated the ability to oxygenate and flow human red blood cells. Thus, by implementing cellularity, biomaterials, and precise organization using the enabling technology of bioprinting, the researcher generated structures that would not be possible otherwise.

19.9 Future perspective

Today's strategy for tissue engineering predominantly depends upon autologous cells from the diseased organ of the patient. In the case of extensive end-stage organ failure, a tissue biopsy may not yield enough normal cells for culture and ultimate transplantation. Primary autologous human cells cannot always be expanded from a particular organ, as is the case with the pancreas. In these cases, pluripotent or highly multipotent human cells could become a source from which the desired tissue can be derived. Induced pluripotent stem cells and embryonic stem cells exhibit two properties: the ability to proliferate in an undifferentiated, but pluripotent state and the ability to differentiate into many specialized cell types. Fetal stem cells, harvested from amniotic fluid and placentas, represent another source. These cells express markers consistent with induced pluripotent and embryonic stem cells, such as octamer-binding transcription factor 4 and stage-specific embryonic antigen 4. They are multipotent and able to differentiate into all three germ layers, and they do not form **teratomas**, nor do they spontaneously transdifferentiate into a malignant phenotype. A further advantage is that the cells have a high replicative potential, and they could be stored for future possible use in the patient, without the risks of rejection and ethical concerns.

In addition to cell sourcing, growth factors and other signaling molecules alone or in combination with biological or synthetic matrices may be used for cell proliferation and differentiation, tissue guidance, and regeneration, and thus replace cell-based tissue engineering. Once the growth factors to be delivered have been identified, there are several important criteria to take into consideration. First, the delivery must be controlled, and allow sustained and localized delivery of small amounts of growth factors to the tissue repair site, as systemic administration can cause toxicity in other organs. Further, the release profile must be controlled in ensuring frequent exposure to these factors for a relatively long time to obtain the desired effect. Growth factors typically have short half-lives once they are introduced into the body and are eliminated rapidly. Finally, the process of incorporating the factors must

not involve harsh solvents or high temperatures that might denature or deactivate the proteins. To meet these criteria, the growth factors could be entrapped within or covalently linked to a polymer matrix and released by matrix degradation.

As the diagnosis of genetic diseases has become extremely accurate, gene editing techniques may be used to overcome some of these challenges as they relate to organ dysfunction. Gene editing techniques could also be used to manipulate cells during the growth and expansion state *in vitro*, prior to creating new tissues and organs using engineering techniques. These strategies could also be utilized in newborns at an earlier stage in order to repopulate the body with genetically modified cells that can subsequently replicate, effecting changes long-term.

Summary

- The engineering of architecturally less complex flat structures (e.g., skin) has been achieved, as has the construction of more complex tubular structures (e.g., blood vessels), and hollow nontubular organs (e.g., bladder and vagina). However, engineering functional solid organs (e.g., kidney, liver) is the main challenge facing the future of the field.
- A number of engineered tissues are currently undergoing clinical trials in patients.
- Today's strategy for tissue engineering predominantly depends upon autologous cells from the diseased organ of the patient.
- Primary autologous human cells cannot always be expanded from a particular organ, as is the case with the pancreas. In these cases, stem cells can become a source of cells from which the desired tissue can be derived.
- Approximately 80% of patients typically on a transplant wait list require a kidney. To develop a bioartificial kidney, some researchers are pursuing the replacement of isolated kidney function parameters with the use of extracorporeal cellular units, while others are aiming at the replacement of total renal function by tissue-engineered bioartificial structures.
- As in renal replacement strategies, the complexity of liver tissue engineering has driven some effort toward liver replacement therapies with BAL replacement, but so far, human studies have been equivocal. New advances in decellularization technology are moving researchers closer to viable *in vitro* constructs.
- Studies into gastrointestinal tissue engineering have demonstrated that SIS, AlloDerm, and other unseeded scaffolds can develop structural features of the normal intestine when implanted in the bowel and may provide an off-the-shelf option for replacement patches for intestinal tissue. Additional studies have shown that cells obtained from intestines, seeded on a scaffold, and formed into a tubular shape developed tissues similar to those in the normal small bowel and have been successfully anastomosed to the native bowel in animal studies. More recent studies have demonstrated promising advances in enteric neuronal function, including internal anal sphincter constructs.
- The most effective therapy for diabetes, since the discovery of insulin by Banting and Best in 1921, has been insulin replacement therapy, but this therapy does not match insulin levels appropriately to the blood glucose concentration. The ultimate goal, therefore, is to develop a permanent endogenous replacement for the lack of insulin, and many tissue engineering strategies have been directed toward this goal.

Classical experiment

After several reports in the past about successful culture of animal and human urothelial and bladder smooth muscle cells, it was only in the 1990s that Southgate et al.¹⁷ characterized in detail cultured human urothelial cells and were able to induce urothelial stratification in vitro. They summarized their research as follows:

The purpose of the work was to establish urothelium as an in vitro model for the study of proliferation, stratification, and differentiation in “complex” epithelia. Normal human urothelial cells were cultured in a serum-free medium. The effects of epidermal growth factor (EGF), cholera toxin (CT), extracellular calcium, and 13-cis-retinoic acid on cell growth, morphology, phenotype, and cytodifferentiation were studied using phase-contrast microscopy and indirect immunofluorescence. Stratification-related changes were additionally analyzed by transmission electron microscopy. Under optimized conditions, long-term cultures were successful in 44 (74.5%) out of 59 specimens. Bacterial infection was the most common cause of failure (nine cases).

Primary urothelial cells required an initial plating density of 1×10^4 cells/cm² for survival; passaged cells survived much lower plating densities (2.53×10^2 cells/cm²). CT significantly improved cell attachment, but neither CT nor

EGF was essential for growth. By contrast, cells failed to proliferate without bovine pituitary extract. In media containing bovine pituitary extract, CT, and EGF, cultures had a mean population doubling time of 14.7 ± 1.8 h, maintained a nonstratified phenotype, and expressed the cytokeratin (CK) profile of basal/intermediate urothelium: CK7, CK8, CK17, CK18, and CK19, with variable expression of CK13. CK20 was not expressed in vitro. CK14 and CK16 were also expressed, suggestive of squamous metaplasia in culture, which could be inhibited with 13-cis-retinoic acid. Increasing extracellular calcium from 0.09 to 0.9–4.0 mM slowed down cell proliferation, induced stratification and desmosome formation, and increased expression of E-cadherin. High calcium, EGF, CT, and retinoic acid did not induce markers of late/terminal urothelial cytodifferentiation.

The above-mentioned study describes a simplified technique for the isolation and long-term culture of human urothelial cells. Urothelial cells in vitro are capable of rapid proliferation and can be induced to form integrated stratifying cell layers in high calcium medium.

Southgate J, et al. Normal human urothelial cells in vitro: proliferation and induction of stratification. *Lab Invest.* 1994;71,583–594.

State-of-the-art experiment

In this study, transplantable urinary bladder neoorgans were reproducibly created in vitro from urothelial and smooth muscle cells grown in culture from canine native bladder biopsies and seeded onto preformed bladder-shaped polymers. The native bladders were subsequently excised from canine donors and replaced with the tissue-engineered neoorgans. In functional evaluations for up to 11 months, the bladder neoorgans demonstrated a normal capacity to retain urine, normal elastic properties, and histological architecture. This study demonstrated, for the first time, that successful reconstitution of an autonomous hollow organ is possible using tissue engineering methods.¹² Patients with end-stage bladder disease can

be treated with cystoplasty using gastrointestinal segments. The presence of such segments in the urinary tract has been associated with many complications. The authors explored an alternative approach using autologous engineered bladder tissues for reconstruction.

Seven patients with myelomeningocele, a birth defect in which the backbone and spinal canal do not close before birth leading to loss of bladder or bowel control, aged 4–19 years with high-pressure, or poorly compliant bladders, were identified as candidates for bladder repair. A bladder biopsy was obtained from each patient. Urothelial and muscle cells were grown in culture and seeded on a

Continued

State-of-the-art experiment—continued

biodegradable bladder-shaped scaffold made of collagen, or a composite of collagen and polyglycolic acid. About 7 weeks after the biopsy, the autologous-engineered bladder constructs were used for reconstruction and implanted either with or without an omental wrap. Serial urodynamics, cystograms, ultrasounds, bladder biopsies, and serum analyses were done. Follow-up range was 22–61 months with a mean of 46 months. Postoperatively, the mean bladder leak point pressure decrease at capacity, the volume, and compliance increase were the greatest in the composite engineered bladders with an omental

wrap. Bowel function returned promptly after surgery. No metabolic consequences were noted, urinary calculi did not form, mucus production was normal, and renal function was preserved. The engineered bladder biopsies showed an adequate structural architecture and phenotype. This study showed that engineered bladder tissues, created with autologous cells seeded on collagen–polyglycolic acid scaffolds, and wrapped in omentum after implantation, can be used in patients who need cystoplasty² (Figs. 19.9 and 19.10).

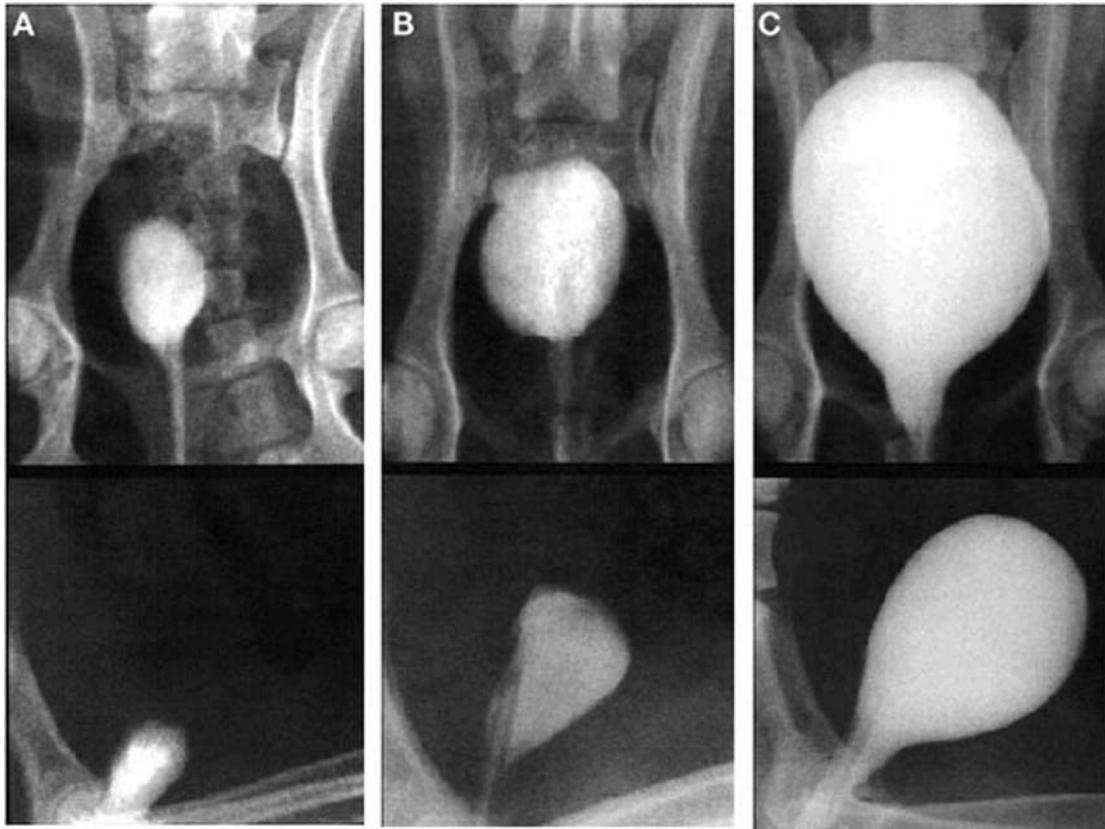
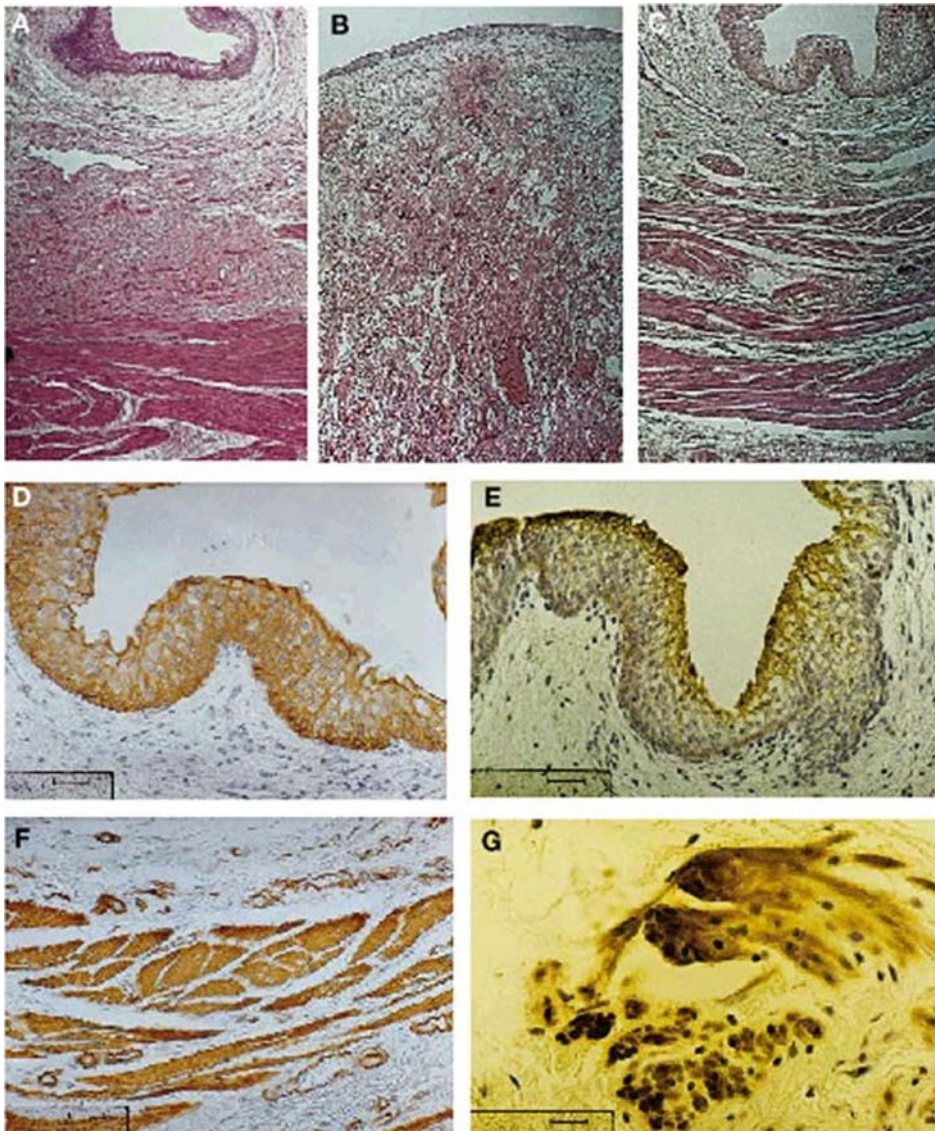


FIGURE 19.9

Radiographic cystograms 11 months after subtotal cystectomy. (a) Subtotal cystectomy without reconstruction (group A), (b) polymer-only implant (group B), and (c) tissue-engineered neoorgan (group C). From Oberpenning F, et al. *De novo reconstitution of a functional mammalian urinary bladder by tissue engineering*. Nat Biotechnol. 1999;17:149–155.

State-of-the-art experiment—continued

**FIGURE 19.10**

Histological and immunochemical analyses of implants 6 months after surgery. Hematoxylin and eosin histologic results are shown for (a) normal canine bladder (group A), (b) the bladder dome of the cell-free polymer reconstructed bladder (group B), and (c) the tissue-engineered neorgan (group C). Immunocytochemical staining of tissue-engineered neorgan revealed; (d) positive staining of the epithelial cell layers with pancytokeratin AE1/AE3 antibodies; (e) urothelial differentiation—related membrane proteins, which constitute the apical plaques of the asymmetric unit membrane in normal urothelium; (f) anti- α smooth muscle actin staining of phenotypically normal smooth muscle; and (g) positive staining with S-100 antibodies binding to neural tissue and nerve sheaths. From Oberpenning F, et al. *De novo reconstitution of a functional mammalian urinary bladder by tissue engineering*. Nat Biotechnol. 1999;17:149–155; Atala A. et al. *Tissue-engineered autologous bladders for patients needing cystoplasty*. Lancet. 2006;367:1241–1246.

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19.11 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
1. List current strategies to overcome organ agenesis and malfunction. What are the pros and cons of these strategies?
 2. What are the different types of biomaterials used for the construction of artificial organs? List their advantages and disadvantages.
 3. Name the cell sources currently being used in organ reconstruction. Are they adequate? What is missing?
 4. Describe the bladder augmentation surgery outlined in this chapter, what were the results of the study? Did the bladder augmentation work?
 5. What are acellular grafts? When was the first time they were used and are they successful?

6. Describe two tissue engineering strategies designed to repair kidney malfunction. What are the challenges associated with these strategies that are preventing these strategies to become available to patients?
 7. Is germ cell renewal in ovaries restricted to fetal life? Consult current literature and discuss the current debate.
 8. What is bioink? How is it being used in engineering of organs?
 9. Summarize the functions of insulin-producing β cells; list the criteria an in vitro engineered β cell should obey to be able to regulate glucose metabolism in the body.
 10. What is SLATE printing? How has it been used in tissue engineering?
 11. What are gene editing techniques? How can they be used to overcome some of these challenges associated with generation of artificial organs?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations.
1. After reading this chapter and in your opinion, what is the biggest challenge currently facing tissue engineers in engineering an artificial organ? Is it complexity of cell types, vascularization, tissue maturation, or something else?
 2. Discuss the commonalities and differences in techniques and concepts in organ tissue engineering.
 3. What are the limiting factors for successful in vivo implantation of in vitro engineered organs?

Challenge-based learning

A bioprinted liver lobule to test novel antifibrotic therapies

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

Liver cirrhosis is the end-stage of chronic liver disease, which is fatal in many cases. The progression of chronic liver disease starts with inflammation, then fibrosis, cirrhosis, and finally liver failure. Pharmacological treatments exist and can be used to mitigate and slow down the progression of the disease but cannot cure it. If pharmacological treatments do not work, then liver transplant is the final option. It is well documented that liver transplantation is the best treatment for end-stage chronic liver disease. However, there is a shortage of donors and the number of patients added to the waiting list grows by the day. The long-term vision in the treatment of chronic liver

disease is to develop effective and noninvasive bio-engineered strategies that aim to reverse the progression of chronic liver disease at the fibrosis stage.

Motivation and stakeholders

Fibrosis is a pathological wound healing in which connective tissue replaces normal parenchymal tissue to the extent that it goes unchecked, leading to considerable tissue remodeling and the formation of permanent scar tissue. Fibrosis occurs in many parts of the body and pharmaceutical strategies are able to reduce tissue fibrosis by specifically targeting molecular and cellular targets of the process. Successful treatment of liver fibrosis requires a liver-specific strategy. Solutions to mitigate liver fibrosis should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as hepatic surgeons, internal medicine doctors, organ systems and biological engineers, and biomanufacturing specialists.

Continued

Challenge-based learning—continued

Problem definition

Current cellular systems to test antifibrotic drugs employ 2D cultured fibroblasts or use animal models. Conventional cell culture lacks the physiological complexity in which liver fibrosis occurs, and animal experimentation is ethically undesirable and animal physiology does not always recapitulate human metabolism. In order to understand and cure the pathophysiology of liver fibrosis, more physiological systems are needed that closely follow the architecture of the liver lobule. Advances on liver systems engineering could be twofold: fidelity in mimicking the architecture and function of the liver lobule and preventing the use of animal models, whose results are difficult to translate to humans. Therefore, there is a need to bioengineer the liver lobule by merging the current knowledge on organ systems to create new therapeutics for liver fibrosis.

Challenge

To generate a bioprinted liver lobule with its vasculature (central vein) to understand the progression and treatment of liver fibrosis with the use iPSCs as cell source. Focus on the spatiotemporal location and activation of liver cells like hepatocytes, fibroblasts, Kupffer, and endothelial cells.

Learning framework

Reading the Organ Tissue engineering chapter and additional literature on liver physiology will help you to understand the following:

1. The anatomy, histology, and physiology of the liver.
2. The pathophysiology of chronic liver disease.
3. The architecture of the liver lobule.
4. The cellular and molecular aspects of fibrosis.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

5. State-of-the-art treatments in liver fibrosis treatment
6. Current available biofabrication methods for organ systems.
7. Cell and biomaterial sources used for bioprinting of liver lobules.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

19.12 Glossary

Allogeneic means taken from different individuals of the same species.

Autologous refers to cells or tissue obtained from the same individual.

Bioartificial is combining a biomaterial with cells to replace a failing organ or tissue.

Biocompatible a substance that is not harmful or toxic to living tissue.

Bioink is a carrier material used to produce an engineered tissue using 3D printing.

BrdU (5-bromo-2'-deoxyuridine) staining is a technique used to identify proliferating cells

Congenital pertains to a condition or defect present at birth.

Dysplasia is any of various types of abnormal growth or development of cells or organs, and the abnormal histology or anatomical structure resulting from such growth.

Extracorporeal is a medical procedure performed outside the body

Hypoplasia is underdevelopment or incomplete development of a tissue or organ.

Hypospadias is a male birth defect where the urethral opening is not located at the tip of the penis.

Iatrogenic is a disorder or illness caused during medical treatment.

Involute refers to turn or roll inward.

Mayer–Rokitansky–Küster–Hauser syndrome is a poorly developed uterus and vagina with normal ovarian function and external genitalia.

Metabolic homeostasis is stable and reproducible metabolic activity in a person or animal.

Myelomeningocele is the most serious type of spina bifida where a sac containing part of the spinal cord, cerebrospinal fluid, and nerves protrudes through the back.

Neooorgans are final structures produced after transplantation of constructs consisting of endogenous stem/progenitor cells grown ex vivo within predesigned matrix scaffolds.

Neurogenic bladder is the lack of bladder control due to disease or injury to the central nervous system.

Omenta (Omentum) are layers of peritoneum that covers abdominal organs.

Organ agenesis is the failure of an organ to develop during embryonic growth and development due to the absence of primordial tissue.

Organoids are complex three-dimensional cultures derived from tissue or pluripotent stem cells to mimic an organ of interest.

SLATE printing is an acronym for Stereolithography Apparatus for Tissue Engineering (SLATE) where living cells are injected into soft gels that contain very small and intricate blood vessels.

Stenotic is a constriction or narrowing of a duct or passage.

Syngeneic is genetically identical or immunologically compatible.

Systemic refers to affecting the body as a whole.

Teratomas are tumors made up of several different types of tissue, such as hair, muscle, teeth, or bone.

Xenogeneic a tissue or organ that is derived from, originating in, or being a member of another species.

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Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Product and process design: scalable and sustainable tissue-engineered product manufacturing

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Abbreviations

ATMP Advanced Therapy Medicinal Product

CAT Committee for Advanced Therapies

CHMP EMA's Committee for Medicinal Products for Human Use

CQA Critical Quality Attribute

DoE Design of Experiments

ELISA Enzyme-Linked Immunosorbent Assay

EMA European Medicines Agency

GMP Good Manufacturing Practice

MA Material Attribute

PP Process Parameter

QbD Quality by Design

QTPP Quality Target Product Profile

TEP Tissue-Engineered Product

UO Unit Operation

20.1 Learning objectives

After reading this chapter you will be able to:

- Recognize the main regulatory bodies and their role.
- Understand what quality is, and why it is the focus of TEP manufacturing.
- Know the meaning of a Unit Operation, a Process Parameter (PP), a Material Attribute (MA), and a Critical Quality Attribute (CQA), and how together they define the manufacturing process. Know the Unit Operations involved in a typical manufacturing process and be able to give concrete examples.
- Be able to explain the added value of Quality-by-Design for manufacturing process development.
- Understand the added value of digital twins for process development and monitoring and control. Know what a Factory-of-the-Future is, and how digital twins contribute to it.

[...] if you haven't manufactured the new thing in substantial quantities, you have not innovated.

J. Gertner, The Idea Factory: Bell Labs and the Great Age of American Innovation, 2013.

All models are wrong, but some are useful.

G. Box, 1980.

20.2 Introduction

Within this chapter, a global overview of product and process design from an industrial manufacturing perspective will be given. Because the technical aspects such as related process and product requirements, the biological mechanisms of action, the dedicated hardware setups, etc., are unique for each **tissue-engineered product (TEP)**, these topics will not be elaborated on. Relevant and application-specific information on this topic can be found in the other chapters of this book. Instead, this chapter aims to introduce several governing concepts that are imperative to the development as well as the future of industrially relevant manufacturing processes for TEP. For example, the **quality by design (QbD)** framework for process development, the use of **digital twins**, as well as certain legal and regulatory aspects related to product manufacturing and quality. Early implementation of a number of manufacturing-related aspects within the development pipeline can be beneficial for the translation of a protocol to a manufacturing process; thus, these aspects will be discussed briefly too. Manufacturing of TEP is closely governed by an extensive set of regulations, directives, and guidelines. Therefore, the legal definitions of TEPs, and **advanced therapy medicinal products (ATMPs)**, of which TEPs are a subclass, provide for a relevant point of reference.

20.2.1 ATMPs—definitions and current manufacturing status

The European Medicines Agency (EMA) identifies ATMPs according to Regulation (EC) 1394/2007 into three main types (Fig. 20.1)¹:

- **Gene therapy medicinal products (GTMPs)**: these products contain genetic information that results into a therapeutic or diagnostic effect. Their mechanism of action is based upon the insertion of “recombinant” genes into the body. Targeted indications can include (but are not limited to) various genetic disorders, cancer, or long-term diseases. Importantly, vaccines against infectious diseases are

ATMP categories				
Purpose	GTMP	sCTMP	TEP	CATP
	Gene therapy medicine products	Somatic-cell therapy medicinal products	Tissue-engineered products	Combined advanced therapy products
	Recombinant nucleic acid of biological origin	Cells or tissues that have been manipulated to change their biological characteristics or not intended to be used for the same essential functions in the body	Cells or tissues issued from tissue engineering that have been manipulated or not intended to be used for the same essential functions in the body	One or more (implantable) medical device(s) and viable cells/tissue or non viable cells/tissue susceptible to have an essential action on human body compared to the previous cited devices
Purpose	Therapeutic, prophylactic or diagnostic effect Regulate, repair, replace, add or delete a genetic sequence	Cure, diagnose or prevent diseases	Repair, regenerate or replace human tissue	Therapeutic, prophylactic or diagnostic effect Repair, regenerate or replace human tissue

FIGURE 20.1
ATMP classification (EU, EMA—CAT).

excluded from this category.² As an example, an RNA-based therapeutic vaccine against cancer is classified as a gene therapy medicinal product, while an RNA vaccine preventing a viral infection is excluded from the ATMP regulation. Kymriah® (against blood cancer), Yescarta® (against B-cell lymphoma), and Zolgensma® (spinal muscular atrophy) are examples of GTMPs.³

- **Somatic-cell therapy medicinal products (sCTMPs):** these are products containing cells or tissues which have been manipulated in such a way that their biological characteristics have been altered to exert a function not native to the original cell identity. Their application can be related to a therapeutic as well as to a diagnostic or preventive action. Any type of therapy in which stem cells are acquired from the patient and differentiated in vitro in order to perform a specific function post-implantation can, for example, be categorized as such. Another example of an sCTMP which also shows the complexity in categorization of ATMPs are dendritic cells (derived in vitro from monocytes) in which mRNA is added to elicit a specific immune response. As the mRNA in this case is considered as a tool to condition the dendritic cells and will have little to no functional contribution postimplantation, and the genotype of the cells will not be altered, such products are also classified as sCTMPs. Alofisel®, Zalmoxis®, and Strimvelis® are examples of approved sCTMP.^{3,4}
- **Tissue-engineered medicinal products (TEMPs):** these products contain cells or tissues that have been modified so they can be used to repair, regenerate, or replace damaged human tissue. Although the distinction between TEPs and sCTMPs is sometimes difficult to make, the difference in intended application is essential in the categorization here. Holoclar®, Spherox®, and ChondroCelect®⁴ are examples of TEPs although the marketing approval for ChondroCelect® has been withdrawn when TiGenix NV notified the European Commission of its decision to discontinue the product due to commercial reasons.⁵

In addition, some ATMPs may include one or more medical devices as an integral part of the medicine, referred to as *combined ATMPs*. An example of this is cells embedded in a biodegradable matrix or scaffold. However, it needs to be mentioned that the presence of a medical device such as a scaffold structure as such is not sufficient to designate a classification as a combined ATMP. It also depends on the actual function of the medical device, either as an integral part of the final product (resulting in a combined product) or as part of the final formulation. In such a case, the medical device should be considered as an “excipient” and therefore as noncombined. For example, when the medical device was used during the development of the product as an initial support structure but does not actively contribute to its therapeutic effect, it is considered as noncombined. More information on the classification of the different ATMPs can be acquired through the documentation provided by EMA.²

Over the past decade, ATMPs have gained regulatory approval and clinical recognition. Several cells, genes, and TEPs have been authorized to be used in patients of which 15 were approved for use in the EU and 9 in the US. 7 out of these 15 products were authorized to be used in both regions as of May 31st, 2020.⁶

However, aside from these products, there have also been preclinical, clinical, and regulatory failures as well as market withdrawals for commercial reasons and effectiveness issues. Within the approved ATMPs, TEPs are only represented by 3 products,⁶ and from the first 285 ATMPs registered by the EU, only 6 are currently commercially available to the patients.⁷ This clearly illustrates that there are

still many risks, challenges, and uncertainties that need to be addressed before TEPs can be put on the market in a sustainable way.

Most ATMPs are autologous treatments, and therefore to some degree also personalized, which also requires a certain personalization of the manufacturing process. Moreover, the patient populations for these treatments range from only a few patients per year (e.g., Strimvelis® for ADA-SCID), to therapies with tens of thousands of patients per year (e.g., CAR-T for leukemia). Therefore, a major challenge is manufacturing these breakthrough treatments at an appropriate scale, level of quality, and cost to enable sustainable market access.⁸

20.2.2 Current challenges of TEP manufacturing

The inherent complexity of TEPs demands attention toward a robust manufacturing approach, starting at early R&D stages of the process and product. This will be essential to facilitate their future clinical implementation, as changes to the process in the clinical phase are both difficult and expensive. The final TEP product ranges from a cell suspension that is to be injected at the damaged tissue site, to complex in vitro developed tissue structures. This broad variation presents unique manufacturing challenges specific to each individual TEP concept and application.

A TEP manufacturing process can be broken down into a series of steps. Typically, the very first steps are related to acquisition of the primary cell source, which comes down to performing a biopsy of patients' tissue and (stem) cell isolation (i.e., most autologous therapies), or starting from frozen vials from a frozen master cell bank (i.e., most allogeneic therapies). Consequent steps are very dependent on the specific TEP, but are typically related to cell suspension reformulation, cell expansion, tissue construct formation, and differentiation. The process typically ends with a final product formulation step and appropriate storage. Each of the process steps is considered a **unit operation (UO)**. Currently, these UOs often consist of a series of conventional manual and open manipulations, and therefore heavily depend on operator expertise. Hence, they may be executed suboptimally, are prone to human errors, are uncontrolled, with high risks for contamination, and may contain processing inconsistencies. Those aspects can have a considerable influence on the quality of the therapy as well as its cost.⁹ Additionally, the required operator expertise and the necessary production cleanrooms make these manually executed processes inherently unscalable: it is difficult and costly to ramp up production if necessary.

In order to translate a laboratory-scale produced 3D-engineered construct to a clinically effective and economically viable product, an efficient, robust, and automated manufacturing process that ensures consistent product quality according to regulatory requirements is required.¹⁰

As mentioned previously, within the regulatory framework of TEPs and ATMPs, the manufacturing is one of the most important aspects to facilitate their successful clinical implementation. However, given its importance for the economic viability of the product, we will also elaborate on some other practical, and equally important, manufacturing considerations such as scalability and manufacturing cost. Quality is thus not only an important regulatory and safety aspect, but also of major socio-economic importance because the TEPs need to be affordable and manufacturable at scale to benefit as many patients as possible.

In the following sections, we will cover the definition of a product in the context of manufacturing, the manufacturing process itself, and how it differs from a protocol, as well as the development of robust manufacturing processes and how they are run during the product's lifetime. Finally, a discussion will be presented on what might be the future of TEP manufacturing and the QbD methodology will be introduced as the guiding principle for TEP manufacturing process development.

20.3 Regulatory aspects of TEP manufacturing

The discipline of tissue engineering ultimately leads to the creation of a product that can be used in several ways which is not limited to its therapeutic application but can, for example, also be associated with diagnostics and in vitro screening. In this chapter, we will only focus on therapeutic applications, as they pose the greatest challenges in safety, quality, and cost. However, before we discuss how such products are manufactured, it is important to understand how they must be characterized and described within the existing European legal framework in order to be manufactured. Within this context, it is important to mention that certain aspects of the regulatory framework are region specific (the EU, for example, distinguishes four different types of ATMPs, while the US only recognizes two). More information on these differences can be found in dedicated literature.^{6,11,12}

20.3.1 Legal framework for TEPs

ATMPs in general, and TEPs in particular, are classified as medicinal products by the **European Medicines Agency, EMA**. To quote their website, EMA's core mission is to "*foster scientific excellence in the evaluation and supervision of medicines, for the benefit of public and animal health in the European Union.*" This includes facilitating development and access to medicines, evaluating applications for marketing authorization, monitoring safety of medicines across their entire life cycle, and providing reliable information on them to the broad public.

At the EMA, several committees have been established, such as the Committee for Advanced Therapies and the Committee for Medicinal Products for Human use (respectively the CAT and CHMP). While the CAT's main responsibility is to prepare a draft opinion on each ATMP application submitted for market authorization to EMA, the final marketing authorization is granted by the CHMP. As situating the entire legal framework in relation to the development and approval of novel ATMPs is a complex matter that goes way beyond the scope of this chapter, the reader is referred to alternate sources.⁴

Circling back to manufacturing, the essence of EMA's established legal and regulatory framework, initially established in Directive 2001/83/EC, is the protection of the public health through ensuring quality, safety, and efficacy of authorized medicines. As mentioned earlier, this marketing authorization is granted by the CHMP after review of all relevant information during the clinical development as well as the advice from both the CAT and the Pharmacovigilance Risk Assessment Committee (with respect to the risk management plan). To ensure that the data as well as the manufacturing strategy presented in this application are satisfactory, the CHMP needs to be convinced that the clinical development activities as well as the future manufacturing will comply with the highest standards of quality within the legislation and that the principles of Good Clinical Practice and Good Manufacturing Practice (GMP) are adhered to.

The U.S. Food and Drug Administration and EMA's focus on product quality has resulted in a set of GMP guidelines specific for ATMPs¹³ and the associated manufacturing process. As compliance of all approved medicinal products as well as investigational medicinal products (in clinical trial but prior to market authorization) to GMP is mandatory, this document is key to the regulatory requirements of a manufacturing process. We will briefly summarize some of the key aspects and would like to refer the interested reader elsewhere for more detailed information.¹³

20.3.2 TEP product quality

As product quality is of utmost importance to maintain and ensure safety and efficacy of the manufactured ATMP, the manufacturer is required to install a **pharmaceutical quality system**. In short, this quality system should ensure that the medicinal product is of the quality required for its intended use. With respect to manufacturing, this implies, for example, training of staff and ensuring that the GMP facilities, equipment and their maintenance, and validation scheme meet the requirements set forth in the GMP guidelines as well as the overall traceability of every performed manipulation and the associated consumables, disposables, and raw materials. Furthermore, the pharmaceutical quality system should ensure that the quality control (QC) mechanisms are operationally independent from production (translating in separate teams, equipment, facilities, etc.) and that planned alterations to the manufacturing process can occur in line with the regulatory guidelines. This quality system needs to undergo continuous assessment of its effectiveness and annual reviews are required for authorized ATMPs.

In close relation to the pharmaceutical quality management system, the guidelines also provide an overview of what **quality control** (QC) is required. Although sampling and testing strategy on the process and final product are key in assuring product quality, a much broader QC strategy is required to ensure compliance with the GMP guidelines. This ranges from the required qualification, validation, and maintenance strategy for both the equipment and premises to the raw materials used in the process, the training of staff, as well as the procedures and requirements in relation to the release of a manufacturing batch of the produced ATMP.

In relation to the QC on the process and the product, an important concept introduced in these GMP guidelines is the **risk-based approach**. It acknowledges the varying degree of complexity of ATMPs and their associated manufacturing process. It is therefore stated that a certain level of flexibility is required and permitted so that the manufacturer of the designated ATMP can implement the required measures specific to the dedicated manufacturing process and in function of the associated risk that is to be controlled through each of these measures. Due to the large variability of products, the quality risks associated with each manufacturing process are product specific. The risk-based approach therefore allows, in compliance with the GMP guidelines, to design the process in a way that each potential risk regarding the product safety, efficacy, and quality is mitigated, allowing the manufacturer to guarantee the final quality of the produced ATMP. As an example of the practical implications of this risk-based approach, one could look at cases in which a final release test on the formulated TEP cannot be performed as it requires immediate administration postmanufacturing. In such a case, the control strategy of the manufacturing process could, for example, be designed in such a way that key intermediates and/or in-process controls with a proven relevance to the final product characteristics are tested and

correlated to the CQAs of the final product. These CQAs are again correlated to the quality target product profile which in essence is the definition of the product identity.

20.3.3 Regulation of TEP manufacturing

Although the major focus of the regulatory documents is on the quality of both the process and the product, dedicated requirements for the actual manufacturing process and environment have also been specified in the guidelines. All of these connect to quality and safety at some level, such as requirements on seed lots and cell bank systems, on final packaging of the product and on the reconstitution of the product after batch release. However, some aspects connected to the inherent principles of a TEP, and ATMPs in general, also have a considerable impact on the manufacturing strategies that are worth mentioning explicitly within the context of this chapter.

As a TEP, by definition, contains cells and/or tissues, it is a living material on which terminal sterilization is not feasible. This implies that all production steps should be performed in an aseptic manner to prevent contamination of the process and product. Presence of pathogens could result in process inconsistencies, product failure, host infection, and even the demise of the patient. Several guidelines have therefore been defined that classify different production zones based on different levels of airborne particles and microbial loads. In this classification, a grade A environment is equivalent to a sterile working environment, while grade D represents the lowest classification. As most of the current “state-of-the-art” TEP manufacturing processes are still based on a considerable series of manual, open process steps in which different raw materials as well as process intermediates come into direct contact with the environment, this requires a maximal degree of protection for the process by performing the associated manipulations in a grade A environment. In practice, this can either be translated to the use of a laminar air flow (grade A environment) in a grade B cleanroom or by using a closed grade A environment such as an isolator in a grade D area. Only for supporting process steps related to the preparation of solutions, such as preparation of the culture medium, which can be followed by a sterilizing filtration step, the grade B environment for the laminar air flow cabinet can be reduced to a grade C. All other open process steps, including the final formulation, fill-and-finish, should always be conducted in the most highly controlled environment. Therefore, the closing of process steps using dedicated sterile disposables and bioprocessing equipment together with aseptic connection technology is the way forward to minimize contact between the process and the environment, allowing manufacturing in a grade C environment.

For various TEP concepts, manufacturing is based on autologous cells. An inherent result of this **personalized medicine** approach is that different raw materials are used for each production campaign. Therefore, the risk of cross-contamination must be assessed and mitigated for each process step. As the formation of aerosols, associated with routine open process steps, is considered as one of the primary potential causes of cross-contamination, this implies that segregated premises or production campaign-based “reservation” of the production suites is used for different manufacturing runs. As a line clearance will be required between each ATMP manufacturing run, referring to a structured procedure through which it is ensured that the equipment and working area are free of products, documents, and materials from the prior manufacturing run and that the entire production area is clean, this implies that parallel manufacturing of different products within the same manufacturing area is not feasible, thereby

increasing costs and invested time. To translate this to a concrete example, the classical open manufacturing of an autologous TEP will often consist of several subsequent cell culture and/or differentiation steps connected through brief manual open process steps such as medium refreshment. This implies that the production environment will be vacant for a significant amount of time between each of the open manufacturing steps. Regulations demand strict product separation, to ensure contamination of one product to a different one (or for autologous therapies, from one patient to a different one). For manufacturing processes that contain open process steps, this requires manufacturers to reserve complete cleanrooms for production runs of a single process.

In response, development of a closed system that is organized as a train with aseptic connection technology, even if this would be for a small subset of steps, would be of particular interest to several ATMP facilities.

20.4 The TEP manufacturing process

A manufacturing process can be broken down in a sequence of UOs that yield a product, in this case a tissue-engineered construct. These operations alter the physical, chemical, and biological characteristics of the product in order to transform it from raw materials into a final TEP. As discussed previously, this product is expected to meet certain quality (i.e., safety and efficacy) standards. For TEPs, which can be considered living constructs, these manufacturing processes are often very product (or therapy) specific, highly specialized, and complex, with a lot of room for error at many points in the process.

Additionally, in the case of autologous processes, there is significant variability in the isolated (stem) cells which are used as starting material. For example, when considering cancer patients (hematological malignancies) that may be eligible for autologous CAR-T therapy: some of these patients are terminally ill and at the end of their treatments, which implies that the starting material (the autologous cells that can be isolated from the patient) can be of low quality to start with. As a result, the manufacturing process needs to be designed in such a way that it is robust, which means that for (relatively) small deviations from the manufacturing protocol or raw materials, no significant negative effect on the quality of the end product is expected.

In autologous cell therapy, patient's own cells (e.g., hematopoietic stem cells) are isolated at the start of the manufacturing process and cultivated. Following genetic modification or other substantial manipulations, these cells will be reintroduced to the same patient as a finished product. Thus, the tissue carries the same genetic properties as the patient into whom it is implanted, thereby overcoming graft-versus-host complications. In allogeneic therapy, on the other hand, cells are provided by a donor. Currently, ever-growing, large cell banks are installed to serve multiple patients, allowing the treating physician to select cells with human leukocyte antigen–haplotypes matching those of the individual patients (at least for most patients). The distinction between autologous and allogeneic therapies is important because it implies different demands for the manufacturing process. An autologous process is a personalized therapy, which implies a high variability and low seeding densities due to the fact that the cells are obtained from a specific patient and thus are defined by patient characteristics (age, health, etc.), and the obtained number of cells that can be extracted is relatively low. Thus, a flexible and somewhat personalized expansion process is necessary, as donor variability can result in different expansion

behaviors (due to previous treatments, aging, and/or smoking, for example). These specific requirements could be achieved with disposable bioreactor systems, with the added advantage of avoiding cross-contamination and reduced storage requirements, as production can specifically be tailored to the existing demand.

20.4.1 Unit operations

As mentioned earlier, the TE manufacturing process can be broken down into a set of UOs. A UO consists of any discrete action executed on the raw materials or unfinished product, with the goal of modifying its composition, concentration, volume, or any other physical, chemical, or biological product attribute. Fig. 20.2 shows some of the more common UOs for a typical TE process, here a bone TEP.

The first UO typically acquires cells from some source. In an autologous process, the cells are harvested from a biopsy or leukapheresis of the patient who will also receive the final TEP, while in an allogeneic process the cells could come from a donor or a frozen cell bank. In almost all cases, the number of cells obtained from this step is too low to engineer a clinically relevant volume of tissue (cell numbers $\geq 10^9$ are needed, in contrast to cell biopsy harvests, which are in the order of 10^6 – 10^7 cells). Consequently, a second UO is often to culture and expand the cell population. Expansion of cells is challenging; culture media and conditions are specifically optimized to conserve stem cell phenotype. However, in the final tissue, we require “specialized” cells to build and maintain the TEP. Thus, a third UO usually aims at differentiating the stem cells to their desired cell type (see **subchapter on differentiation**), which depends on the type of TEP that is envisioned (e.g., osteoblasts and chondroblasts for bone and fibroblasts for skin). Finally, the manufactured TE product is to be assessed for safety and efficacy before being implanted in a patient. This is done during QC which consists of a series of tests on the TE product to determine if its key characteristics (e.g., volume, cell identity, cell viability, extracellular matrix (ECM) composition, clonality, etc.) are at the levels we want them to be. If the TE product passes these tests, then it is ready to be implanted in the patient.

The name of the UO here only describes what is happening to the product on a general level. The exact implementation of the UO can still substantially differ from process to process and be defined by UO specific parameters, referred to as **Process Parameters** (PPs): their settings determine the performance

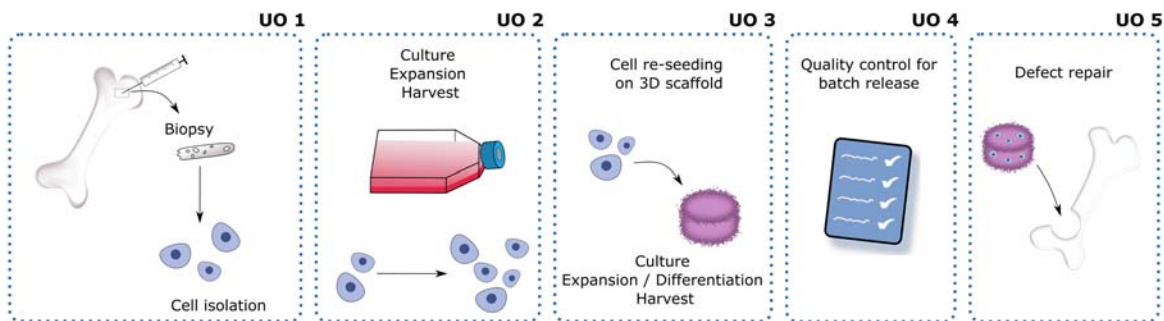


FIGURE 20.2

General manufacturing process of a bone-derived TE product.

(“effectiveness”) of the UO. In addition to the PPs, a Unit Operation also has raw materials with MAs as inputs, and product CQAs as outputs. These outputs of the unit operation are a function of the PP settings and raw MAs that go in it (Fig. 20.6).¹⁴

In a cell seeding UO, for example, cells are deposited on a solid 2D, or 3D surface (also called a scaffold). This deposition can be done manually (e.g., pipetting), or in an automated manner (e.g., with a peristaltic pump in a perfusion system). Additionally, the UO has several parameters that can also vary depending on the process. For example, for the seeding UO, such parameters can include the seeding density and the volume of the suspension (for manual seeding) or the perfusion rate (for automated seeding). For cell expansion on a scaffold (i.e., fixed bed), local nutrient and metabolite concentrations are important variables, and it is important to realize that these local concentrations can be significantly different from the concentrations measured in the “bulk” medium reservoir or elsewhere in the bioreactor. Local nutrient concentrations are affected by the cell density, which varies over time. In turn, the flow regime affects these local concentrations and the viability of the cells (mainly through shear stress). This flow regime is defined by the scaffold geometry, the flow rate, the cell density in the scaffold, and the amount of ECM present. Moreover, the scaffold geometry can change between processes (manufacturing variability of the scaffold) or within the process itself. In this case, 3D-printed scaffolds have a big advantage because there is full control over the scaffold geometry, and it is fully reproducible (see [Chapter 10 Microfabrication technology in tissue engineering](#)). Finally, in case of autologous TEPs, an additional level of complexity is introduced as the influence of all the variables mentioned above can vary significantly from patient to patient.

20.4.2 Process monitoring

Even if it would be possible to execute the process in the same way every time (which is practically only feasible when the process is fully automated), variation in process outcome will still occur due to uncontrolled differences in the raw materials, and potential additional uncontrolled variability from manual and process operations in case the process is not fully automated. Thus, for the process outcome to be more consistent, a monitoring and control strategy needs to be implemented, with the main goal to detect significant variability when it occurs, and to correct the process trajectory accordingly. For example, if the patient is of old age and smokes, it is likely that the doubling time of autologous stem cells will be lower than that of an average patient, resulting in expansion to be less efficient and consequently an expansion step that needs to be extended. Alternatively, donor-to-donor variability, for example, in the glucose metabolism of the cells, would require the medium refreshment strategy to be altered as well. Ideally, the monitoring system quantifies the expansion rate and the glucose consumption rate and adjusts the process accordingly.

Such a monitoring system is formally part of a QC strategy, a required aspect of TE manufacturing from a regulatory standpoint. Minimally, the process monitoring aspect of the QC strategy requires the manufacturer to demonstrate safety and efficacy of the product before it can be implanted in a patient, as discussed previously. However, as mentioned above, the QC strategy and monitoring systems can be much more elaborate and advanced. While a bare minimum QC strategy only quantifies the CQAs destructively, on one timepoint (at the end of the manufacturing process, before product release), an advanced and elaborate QC strategy monitors the CQAs nondestructively and continuously throughout

the process, in addition to other process variables which might be of interest but not directly related to the quality of the product, such as nutrient consumption rates. A process with such an advanced monitoring strategy is inevitably more robust than a process with a basic monitoring strategy, as it can detect and anticipate process deviations and correct for them.

The nondestructiveness of the monitoring strategy refers to the fact that no cells or tissue is destroyed for QC purposes, and thus fully remains available for implantation into the patient. This is beneficial especially for autologous therapies, where the manufactured volume of tissue is relatively small as it's destined for only one patient. For allogeneic therapies, products are mostly manufactured in large batches preparing product for many patients, and so there is more product available that can be assigned to QC purposes.

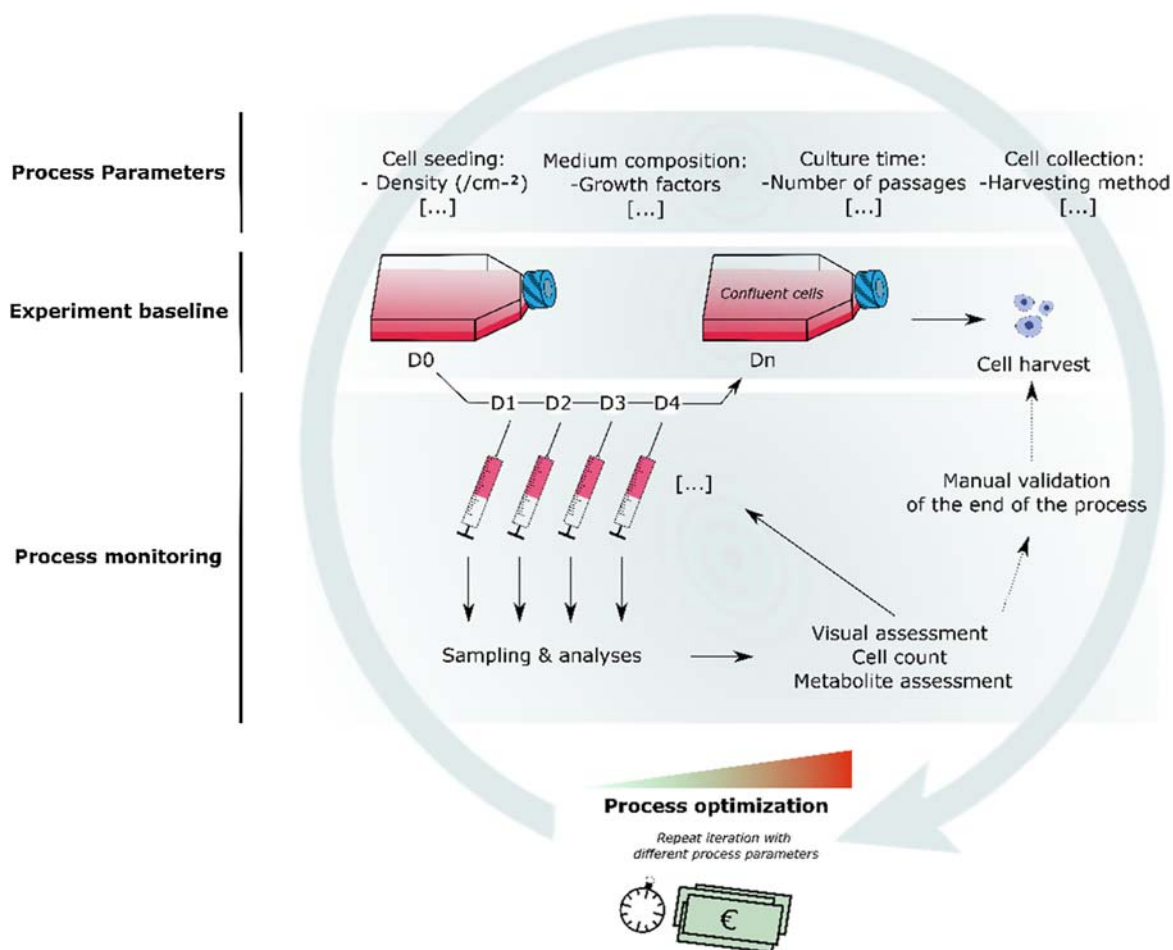
20.4.3 Process scalability and cost

As mentioned previously, process robustness is essential from a quality standpoint, which is essential for patient safety. However, quality is not the only aspect of manufacturing that is important. The scalability and manufacturing costs are essential as well (see [Chapter 13 Bioreactors: Enabling technologies for research and manufacturing](#)). A scalable process is one for which the number of produced batches (e.g., TE constructs) can be increased rapidly and efficiently, if necessary. Scalable processes typically have a high degree of automation and use single-use bioreactors, in contrast to stainless steel systems, which need extensive cleaning between process runs. The manufacturing cost of a process includes raw materials (cells, growth media, reagents), equipment (bioreactors, centrifuges), facilities (cleanrooms, labs), and personnel (operators, regulatory affairs). Obviously, the most ideal manufacturing process is one which is robust, scalable, and cost-effective. However, it is often not possible to maximize all three features independently and some trade-offs must be made. For example, from a process robustness and quality standpoint it can be interesting to use a large culture volume, ensuring nutrients are always in excess and metabolite concentrations are not inhibiting. However, culture medium is often a major cost driver in manufacturing, and thus it can be interesting to minimize the volume of medium that is used ([Figs. 20.3 and 20.4](#)).

20.5 Manufacturing process development: quality by design

As mentioned in the previous section, a TE manufacturing process typically consists of multiple UOs, each with many variables affecting one another as well as the quality of the TEP. To design a process that consistently delivers the required quality (i.e., a robust process), these effects need to be identified. Clearly, product quality is central in TE manufacturing. This product quality consists of several aspects which, when taken together, fully determine the safety and efficacy of the TE product when transplanted in a patient. These quality aspects are defined as CQAs of the product, and they play a central role both in TE manufacturing and process development. [Table 20.1](#) shows some hypothetical CQAs for a bone TE product. Each CQA links with a specific analytical method that quantifies the CQA allowing assessment of the TEP, and its range.

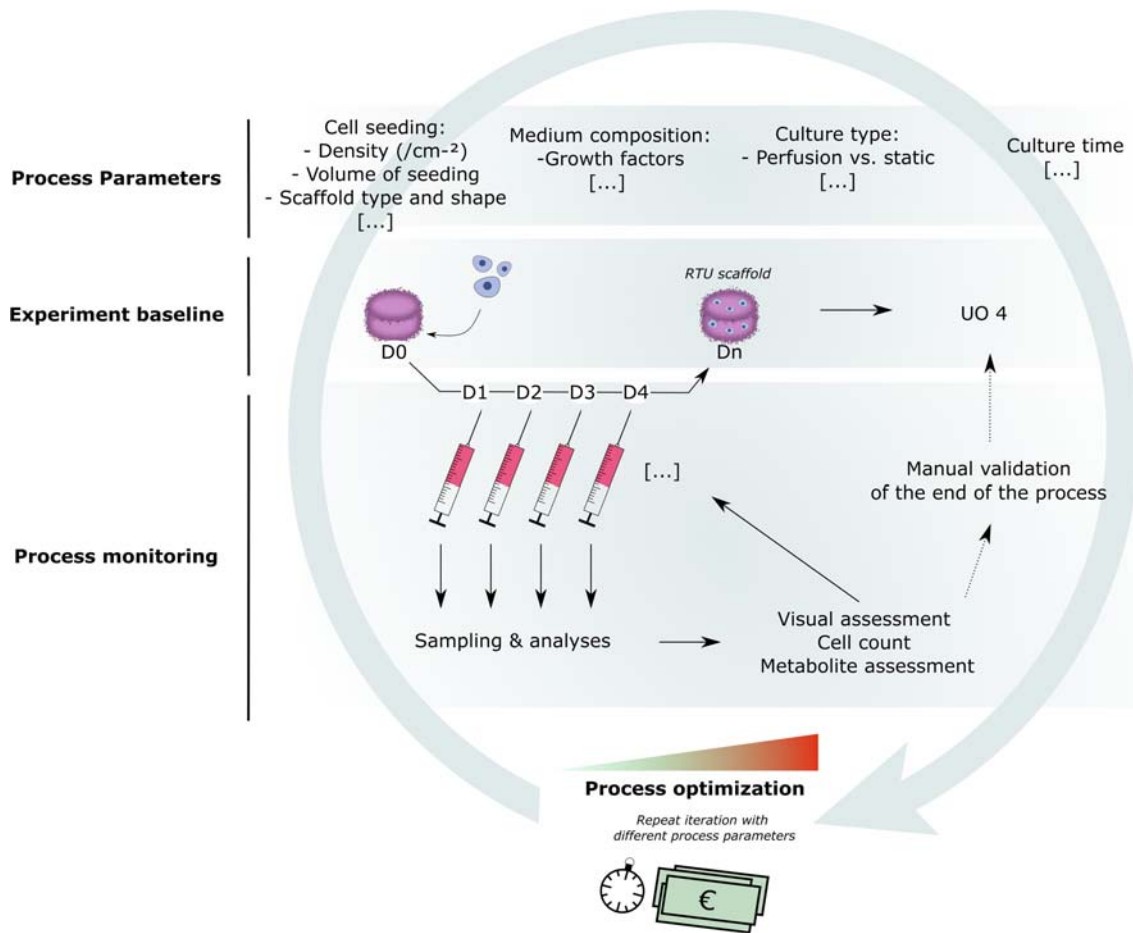
TEPs (and consequently also the manufacturing processes) often are engineered in (academic) laboratory settings, where the TEPs are manufactured by skilled and knowledgeable researchers, through a

**FIGURE 20.3**

Detailed unit operation 2.

complex process involving many manual operations. In the hands of these highly skilled individuals, the process can be robust. However, to have industrial relevance, the process should also be manufacturable at a larger scale, by operators that are not experts.

QbD is a methodology that can be used for process development. It is knowledge and risk-based, which means that it minimizes the risk for the patient and focuses on data-driven decision-making. QbD has originally been employed in other industries, such as the automotive industry; the methodology is equally relevant for biomedical sciences and production of ATMPs, given their complexity. The central assumption in QbD for process development is that the outcome of a UO (and by extension, a process), defined by the CQAs, is determined by, and thus a function of, the MAs and the PPs (see Fig. 20.5).

**FIGURE 20.4**

Detailed unit operation 3.

Table 20.1 Some of the most common critical quality attributes for bone TE products.

Critical quality attribute	Method	Range
Cell density	Histology	>2.5E+07 cells/cm ³
VEGF content	ELISA	35–80 pg/μg protein
Mineralization	Micro-CT	>55%
Volume	Weighing	7.5–11 cm ³

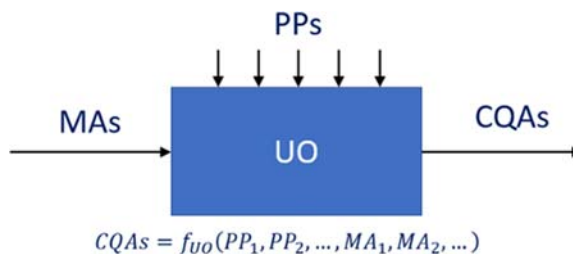
**FIGURE 20.5**

Illustration of the concept of a unit operation.

MAs are the properties of the materials used in the UO, for example, the age of the cell donor or the glucose concentration in the growth medium. The PPs are the settings used to execute the UO, for example, the seeding density of the cells, or the flow rate that is used in a bioreactor (see [Chapter 12 Bioreactors: Enabling technologies for research and manufacturing](#)).

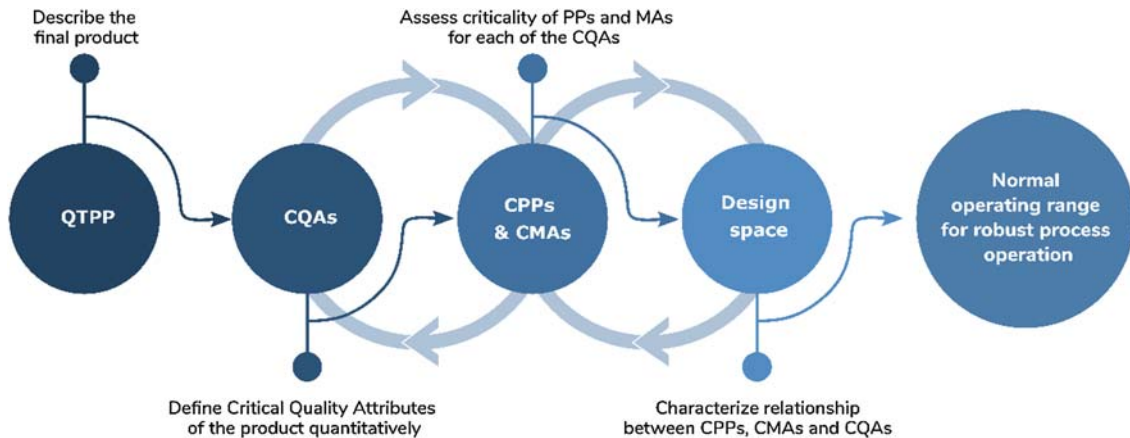
Manufacturing process development through QbD then focuses on the interplay of PPs, MAs, and the CQAs. If this relationship is known, then we know precisely how to execute/adapt the process to achieve the desired quality .

In process development certain aspects (UOs, settings of the PPs, hardware, raw materials) of the process are altered to increase product quality, yield, and robustness. If done correctly, the optimal settings of these parameters are known and can be implemented to yield a well-functioning, performant process. Moreover, the characterization of the relationship between the PPs and the CQAs allows the operators to adjust the process when needed and to anticipate on donor-to-donor variation in cell performance. This is referred to as process robustness, a key feature of an optimized manufacturing process that a manual protocol originating from early development will lack.

In preparation of the experimentation, a risk analysis is performed. These analyses serve to assess the current state of the process and the criticality of the different components (UOs, PPs, ...) for achieving the desired product quality and thus ensuring patient safety and efficacy. From these risk analyses, certain UOs and PPs will result as most critical (i.e., the highest risk). These UOs and PPs are then studied and optimized experimentally.

20.5.1 Design-of-experiments

The characterization of these relationships is done through experimentation and data-based modeling. For example, if process development engineers aim to assess the effect of flow rate, glucose concentration, and scaffold porosity on the growth rate of cells in the expansion step, dedicated experiments can be set up in which these three variables are varied, and the growth rate of the cells is measured. A statistical model can then be built from this data which describes the effect of these variables on the growth rate of the cells, and this model can also be used to estimate which flow rate, glucose concentration, and scaffold porosity is optimal to achieve the required cell growth rate. As mentioned previously, in the case of TE, there are often a lot of these variables that affect one another.

**FIGURE 20.6**

Quality by design workflow.

Design-of-Experiments (DoE)¹⁷ is a multivariate approach to both experimentation and statistical analysis, especially suited to deal with the complexity in TE process development. An additional advantage of DoE is efficiency: it allows a researcher to collect the maximum amount of information from a limited number of experiments. The goal of DoE is two-stage:

1. Define for each UO, which MAs and PPs have the largest effect on the CQAs
2. Determine the optimal settings (or acceptable ranges) for these MAs and PPs to achieve the desired quality

Once this is achieved for all UOs, the process is considered fully optimized. The settings that are determined in the second stage then form the control strategy for the process. The next step would then be to validate the process, according to GMP standards (Fig. 20.6).

20.6 Smart manufacturing driven by digital twins

With more and more ATMPs reaching the market, and even more in the pipeline, production of ATMPs must move from a laboratory craft exerted by highly trained personnel, toward closed and automated manufacturing processes. The latter reduce operator-induced variability, risk of contamination, and manufacturing costs, and will allow to reach more patients at an affordable price. Additionally, personalized medicine is pushing boundaries even further by tailoring the therapy to the individual. Here, the product is adapted to fit the needs of individual patients. For example, a bone TEP can be engineered to perfectly fit an individual's bone defect, personalized in volume and shape, resulting in better patient care and quality of life. These personalized manufacturing processes require additional process flexibility and significantly increased process control capability, as the operating range of the process is in that case much bigger. Thus, such processes require significant process knowledge and flexibility, which makes a QbD approach for process development a necessity.

Additionally, it is a challenge to monitor and control the manufacturing process and to consistently guarantee product quality. For example, for large-batch production of TEPs, it is possible to perform destructive measurements on a small fraction of the batch to verify that all products meet the required

quality standards. However, this is not possible for personalized manufacturing since every product is unique and intrinsically limited in amount, and therefore alternative methods are needed to monitor and control the manufacturing process noninvasively.

Industry 4.0 (the fourth industrial revolution) is currently revolutionizing manufacturing processes by the implementation of innovations that turn traditional practices into so-called smart factories.¹⁸

These innovations include the automation and digital transformation of manufacturing, the acquisition and analysis of process data, and the communication between different machines. This results in self-managing systems that can monitor a manufacturing process in real-time and use these data, together with historical data and fundamental knowledge of the process, to predict and optimize the process outcome.

A clear example of this digital transformation in the ATMP production process is the development of **soft sensing**.¹⁹ In this method, process data that are easily obtained by hardware sensors are combined with machine learning (ML) software models to monitor or predict difficult-to-measure key process states, such as CQAs. For example, nutrient and metabolite concentrations like glucose and lactate are monitored during a cell culture UO and correlated with the number of cells as shown in Fig. 20.7. The glucose and lactate concentration that can be measured online can serve as a soft sensor for the number of cells in the culture. Therefore, if such soft sensors are developed successfully, they are a valuable alternative to destructive biological assays and thus allow for closed and personalized

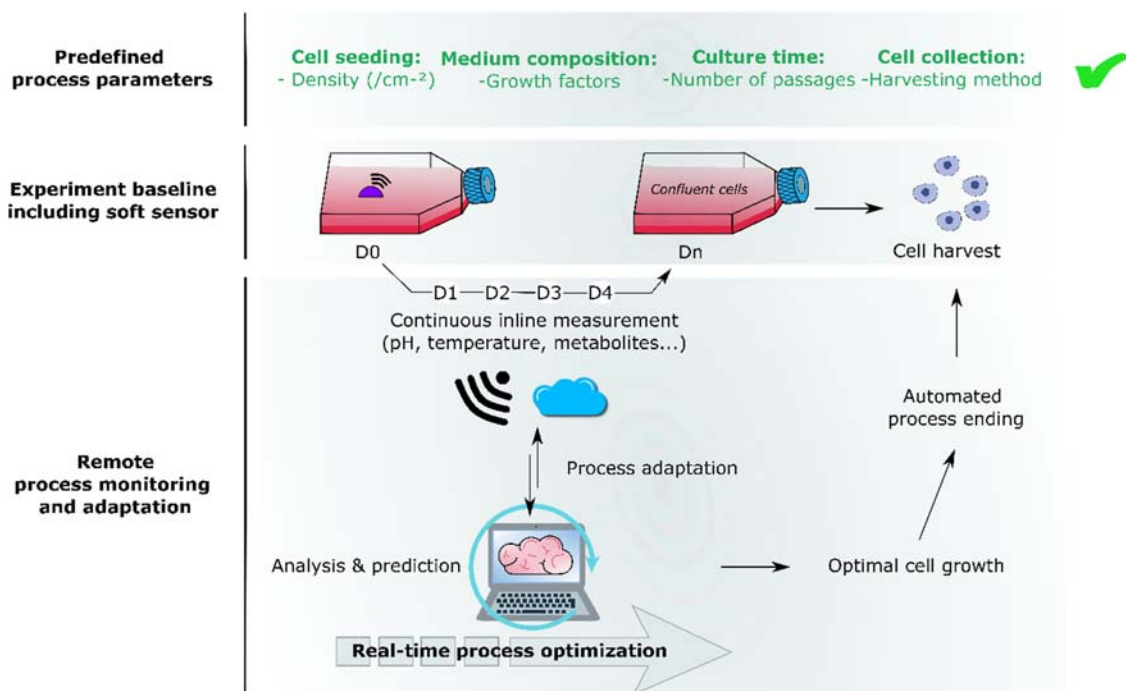


FIGURE 20.7

Role of soft sensors in process monitoring.

manufacturing. In this way, the manual operations during cell culture for medium sampling and refreshment, as shown earlier in Fig. 20.3, can be omitted, resulting in a smaller risk of contamination and disturbances.

Soft sensing is just one example of a large set of digital applications that form an important aspect of smart factories, also known as “digital twins.” A digital twin is commonly described as a virtual, digital replica of a physical product or process. It is a real-time computer model used to predict a process outcome, optimize a process design, or monitor and control a production process. The digital twin receives real-time sensor data of the process and uses historical data or scientifically based assumptions (e.g., physical laws) to simulate the process evolution. Interestingly, besides predicting the outcome of the manufacturing process, a digital twin can be used to assess the effect of process parameter alteration during the manufacturing process in real-time (e.g., bioreactor stirring rate and medium refreshment) and select the optimal conditions for each individual process. In this way, both an increased understanding of the mechanisms underlying a process as well as tools to real-time monitor and adjust a process can be obtained, resulting in increased production efficiency and a higher quality of the final product at a minimized cost.

In addition, digital twins can also play an important role in the design and optimization of the manufacturing process itself. Classically, the development of a TEP manufacturing process via QbD requires multiple iterations in which the effect of PPs on CQAs is investigated through dedicated experiments. As demonstrated in Fig. 20.8, this is an expensive and time-consuming procedure, specifically for TEP manufacturing which is often affected by many PPs.

Digital twins can reduce the cost and time required to develop the manufacturing process by prescreening the effect of PPs on the CQAs and selecting the most promising candidates to be tested in an experimental setting (Fig. 20.8). Obviously, this methodology requires a considerable initial investment for development and validation of the digital twin. However, once validated, the digital twin can significantly reduce the experiments for manufacturing process development and thus fast-track the process development at a reduced cost. Besides, some digital twins are also able to extrapolate beyond the experimental design space and can be used to investigate the effect of upscaling of the manufacturing process, which allows to perform the experiments at a smaller scale and thus further reduces development cost and time.

20.6.1 *In silico* models in digital twins

The computer models that are used in digital twins can be subdivided into mechanistic and data-driven models.²⁰ Both model types have their own strengths and weaknesses, and some digital twins may primarily or even exclusively use one or the other, depending on the application. However, the most successful digital twins typically use a combination of both.

Firstly, **mechanistic models** are directly based on the scientific laws underlying the process.²¹ To illustrate—in a mechanistic model of TE construct growth, culture dynamics could be described by cell behavior (metabolism, proliferation, and death) at the individual or coarse-grained cell level, while convection–diffusion equations could be used to calculate the spatial distribution of nutrients in the bioreactor during culture. The biggest advantage of mechanistic models is that they can provide insight

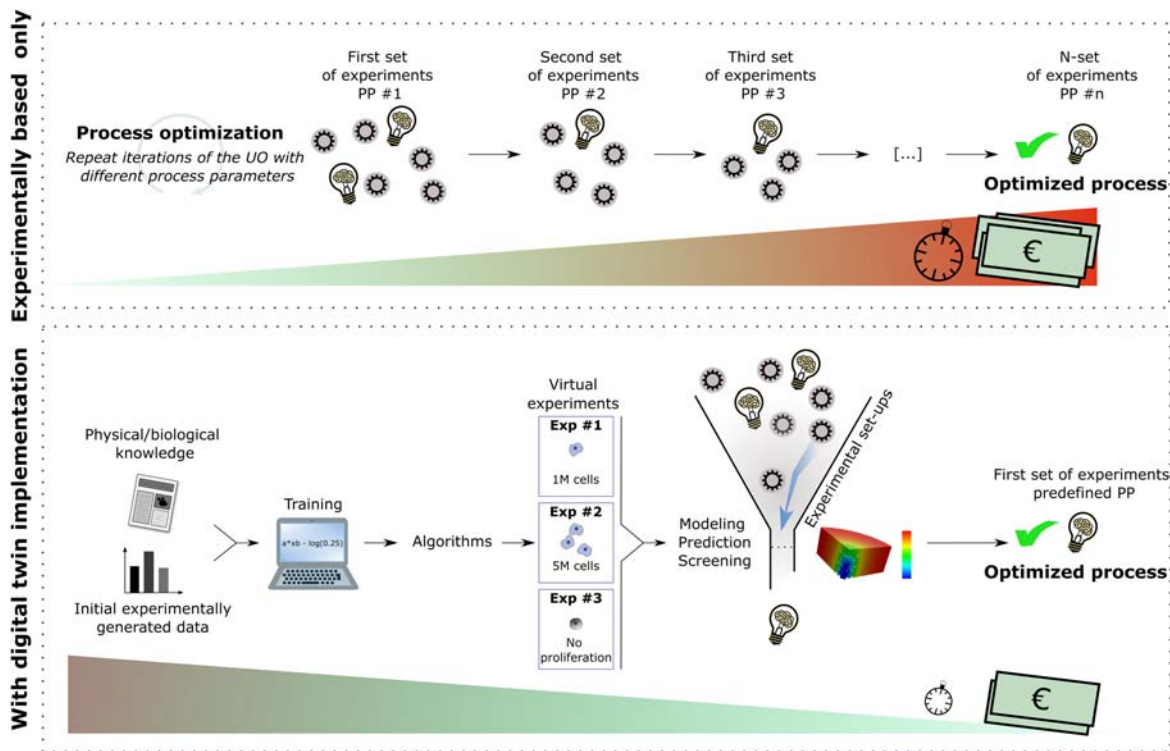


FIGURE 20.8

Process optimization with or without digital twin implementation.

into the process because their parameters are based on the underlying physics. Therefore, a mechanistic model can also be easily adapted to, e.g., a change in the geometry of the TE construct or the culture bioreactor. However, mechanistic models in general have a high cost of development, (some of) their parameters can be difficult to validate, and they may require large computing resources, which might hamper real-time computations.

The data-driven approach uses historical process data and ML models to build (a part of) a digital twin¹⁹. In contrast to a mechanistic model, a **data-driven model** does not rely on physical knowledge of the system, but uses process data to build the mathematical model, such as cell counts, seeding densities, or nutrient consumption rates, primarily correlating different process variables and control signals (e.g., CQA levels). They are sometimes called “black-box” models, as they provide little to no new insight into the modeled system because they are not based on physical or biological knowledge. Consequently, these models cannot extrapolate beyond the range of parameters for which they are trained and therefore cannot deal with process adaptations (e.g., geometry of a TE construct) as easily as mechanistic models. However, a major advantage of this approach is that the training and evaluation, provided that sufficient data is available, is fast and efficient. They can run in real-time with the process and the methodology for their validation is relatively straightforward. The soft sensor described earlier is a clear example of a data-driven digital twin.

Hybrid models are an attempt to combine the benefits of both mechanistic and data-driven models, in which a mechanistic approach is used to model part of the process for which established knowledge is available, while the data-based approach models the parts of the process which are complex and not fully understood.²² For example, a model of TE construct culture could be developed in which the fluid dynamics and nutrient distributions are modeled mechanistically, while the relationship between nutrient concentrations, cell-to-cell contact inhibition, and the growth kinetics of a cell culture is modeled in a data-driven way. Together, both approaches form a hybrid model that could be used in the process development stage to determine the optimal geometry of a TE construct and the culture chamber, which allows efficient nutrient distribution throughout the cell culture. During manufacturing itself, the model could be used to monitor the cell density in the TE construct noninvasively and in real-time.

20.7 Future perspective

Finally, looking back at the concepts discussed in this chapter, the world has taken great strides in enabling tissue engineering (and ATMPs more generally) toward a commercially viable scale. Governments began appreciating the change these medicinal products can bring to patients and rolled out regulations that have been improving over more than a decade. Marketing authorization for ATMPs and their tentative commercial success are bringing optimism to patients, researchers, and investors alike. Nevertheless, TEPs remain complex medicines as they aim to mimic or regenerate the complexity of tissue functionalities. Therefore, the field needs to build on the existing innovations and at the same time keep momentum in developing novel ones to ensure a sustainable patient outcome.

While we are not quite there yet, several of the innovations necessary to enable Industry 4.0 for tissue engineering are in place. Most of them still need further development and fine-tuning to reach technology readiness levels suitable for industrial applications.²⁶ A certain midpoint on the journey to modular factories will be processes configured using currently available bioreactors and up- and downstream processing equipment (e.g., see **20.9 State-of-the-art experiment**). While certain steps still need operator involvement, the crucial unit operations can already be carried out in a controlled and automated manner, reducing errors and increasing reproducibility.

With each new TE product, a certain amount of manufacturing process development is needed at the product development, preclinical, and clinical stages. As most TE products are autologous, we strongly believe that process development needs to be rooted in a risk-based, QbD approach. This is necessary not only to deal with the significant amount of raw material variability that is present for autologous therapies but also to facilitate the desired, deliberate product variation in personalized therapies. The product attributes in personalized therapies are tailored to the needs of each patient.

Despite the process robustness facilitated by QbD, advanced monitoring systems will have to complement the developed processes. The monitoring solutions used here should ideally be nondestructive, automated, and provide real-time continuous read-outs. Besides traditional “hardware sensors,” a lot of promise lies in the use of data-based software models (“soft sensors”) able to infer the process state from readily available process data.

To allow for scaled-out and decentralized manufacturing, disposable, and fully closed systems (such as the Cocoon) will have to replace T-flasks, isolators, and cleanrooms. Disposable systems create the opportunity to adapt the hardware to the process, which is especially interesting for personalized therapies. These innovations can be facilitated by additive manufacturing, which is compatible with on-demand, customized part production.

To make this type of next-generation process run safely, the hardware must be connected to an advanced digital twin, able to adjust the process on-the-go, based on inline, nondestructive readouts. By automatically keeping process parameters in a predefined design space, considering patient-to-patient variability, the safety and quality of the end-product can be ensured.

In education and academia, the emphasis currently lies on the intricacies of the TE products themselves. With this chapter, we hope to give an overview of the different aspects of TE manufacturing and show that there are a lot of important and exciting challenges still to be tackled in this area as well. Without a doubt, powerful and exciting new TE products will be developed in the coming decades. To maximize societal benefit of those therapies, appropriate manufacturing processes should be in place as well (Figs. 20.9 and 20.10).

Summary

- TEP manufacturing combines all aspects required to commercially produce a tissue-engineered product
- From a regulatory standpoint, tissue-engineered products fall under ATMPs, along with gene therapy medicinal products and cell therapy medicinal products
- TEP quality is the focus in TEP manufacturing, directly tied to safety and efficacy for the patient and captured in the CQAs of the TEP
- A manufacturing process is a sequence of unit operations, each with their own set of PPs and MAs
- A generic manufacturing process does not exist, and dedicated manufacturing process development is needed for every TEP
- Quality-by-Design is a process development methodology that is especially suited for development of complex processes, such as TEP manufacturing
- TEPs can be autologous or allogeneic. Most TEPs are autologous, and for these processes, it is important that the monitoring and control strategy is nondestructive
- TEPs processes are often expensive and time consuming. *In silico* models of the process can both help to cut costs, and speed up process development
- The TEP factory of the future is one that facilitates robust, cost-effective manufacturing of personalized TEPs

Classical experiment

NOVOCART® 3D—a tissue-engineered product manufacturing case

A manufacturing process is a sequence of UOs that yields a product.¹⁵ While this chapter primarily focuses on operations taking place in a cleanroom (or bioreactor), it is important to remember that the whole manufacturing chain typically spans many more activities, including but

not limited to starting material procurement (or collection), scaffold manufacturing, QC, and logistics.

As an example to how a TEP is currently manufactured we chose NOVOCART 3D,¹⁶ an autologous product, combining autologous cells with a biologic device that envisions repair of articular cartilage of the knee [Fig. 20.9]. The product is still under investigation and is currently in Phase III clinical

Classical experiment—continued

trials in the US and EU (information obtained on clinicaltrials.gov on May 14th, 2021).

The delivery of NOVOCART to patients consists of three key phases, material collection, the manufacturing process (or processing and/or cell manipulation), and implantation, spread over 3 weeks, and includes two surgeries.

In general, starting material collection may involve a biopsy or blood leukapheresis of a donor. In case of NOVOCART 3D, the biopsy is taken from the knee cartilage, after informed consent is given by the patient. Note that during that stage, donor testing also takes place.

In the processing and/or cell manipulation phase, patient-derived material is transported to a GMP-certified manufacturing facility, where the patients' chondrocytes are isolated and expanded.

When enough chondrocytes are available, the cells are seeded to a decellularized bovine pericardium and a sponge-like collagen matrix (scaffold) and allowed to attach and distribute themselves over the fibers (product manufacturing phase). This combination of cells and scaffold is what constitutes the final TEP.

The manufacturing process is executed in GMP-compliant cleanroom facilities equipped with isolators that meet the requirements of cleanroom class A. All steps in the process are standardized and are continually monitored and documented for quality (identity, purity, and potency) and sterility. By using only tested homologous serum, it is possible to exclude the immunological reactions or the transfer of infections of animal origin. The use of antibiotics and antimycotics is also avoided.

For every procedure, autologous patient cells are treated as a separate manufacturing batch and carefully managed for traceability. Detailed log sheets with concluding test reports on the sterility tests, cellular, and microbiological analyses are prepared for each transplant. The quality of the cells and molecular biology of the transplant are checked (e.g., quantitative PCR) immediately prior to the return of the product to the user. Along with the product, the user receives a concluding test report with these data. Approximately 3 weeks after the harvest of the biopsy, release testing is completed and the NOVOCART 3D implant is shipped to the surgery center, to be transplanted in the patient.

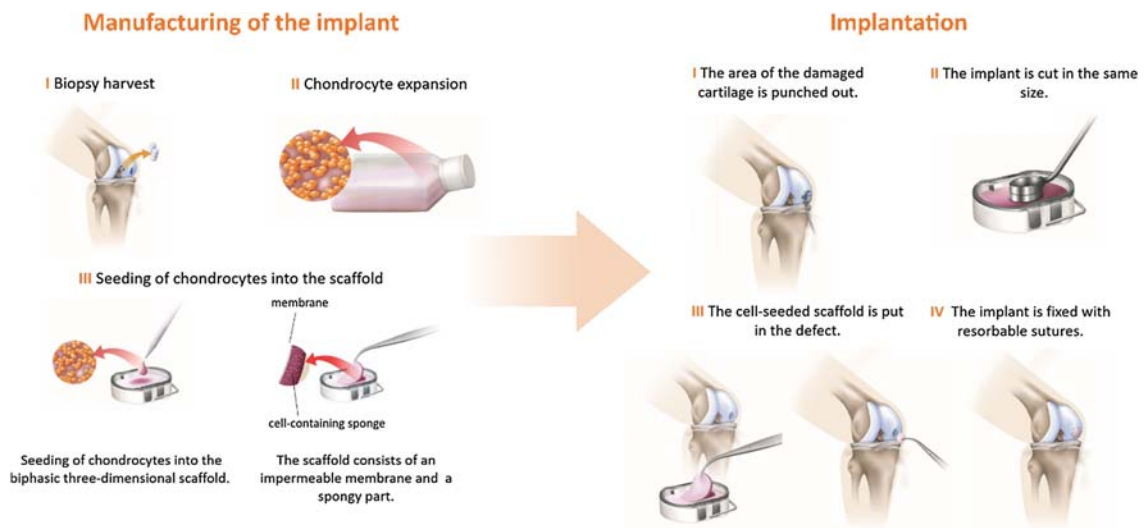


FIGURE 20.9

An illustration of the manufacturing process of NOVOCART 3D. Adapted from NOVOCART 3D, 2021, <https://www.aesculapbiologics.com/en/patients/novocart-3d.html>. NOVOCART 3D, 2021. Available from: <https://www.aesculapbiologics.com/en/patients/novocart-3d.html>.

State-of-the-art experiment

Lonza/OCTANE Cocoon®—a tissue-engineered product manufacturing technology case

A commercially available instrument that combines a number of concepts put forth in this chapter as the way forward for TEP manufacturing, particularly for autologous (or personalized medicine) applications, is the Lonza/Octane Cocoon platform.²³

This platform was developed to enable scalable manufacturing of personalized medicine products (Fig. 20.10). The center of the platform is the disposable cassette that integrates the ability to carry out various up- and downstream unit operations, with a set of in-line sensors (temperature, pH, and dissolved oxygen [DO]). To facilitate out-scaling (parallel production), necessary for large-scale autologous product manufacturing, single cassettes can be mounted on racks to save space and connected into a central control system.

During process development, the cassette is to be optimized/customized to accommodate the process for the TEP in question. QbD can be a viable strategy to guide that process.

The functionally closed nature of executed processes allows to place the devices in a less stringent cleanroom environment, lowering the capital expense for the manufacturing

facility. Built-in automation eliminates the risk of operator mistakes, at least for the unit operations carried out within the cassette.

In its current version, the platform is oriented primarily at gene- and immuno-therapy product manufacturing. It has already been used for point-of-care manufacturing of a CAR T-cell therapy.²⁴ However, the intention is to expand the platform's capacity to tissue-engineered products as well. Notwithstanding, the Cocoon has also been used in this broader context, the caveat being that process development may necessitate the integration of multiple devices to carry out all necessary handlings. Some of the steps may still have to be executed manually. A more extensive validation effort may also be required from a regulatory perspective.

Vukasovic et al.,²⁵ for example, employed a Cocoon system to execute the cartilage digestion phase of a cartilage graft manufacturing process. It is important to note that this was an animal study aimed at assessing manufacturing issues. The cassette integrated a digestion vial, input ports for liquid addition and waste, and product collection compartments. The cell expansion and differentiation phases took place in different units, together with manual operations.



FIGURE 20.10

A visualization of the Lonza/OCTANE Cocoon system. From Octane Medical Group Inc. Cocoon platform, 2021. Available from: <https://pharma.lonza.com/technologies-products/cocoon-platform>.

State-of-the-art experiment—continued

The original process comprised of a series of labor-intensive, manual steps, leaving the product vulnerable to operator variability, as well as making it difficult to scale up.

The long-term vision for the Cocoon is to create facilities where numerous cassettes are integrated into centrally controlled stacks, each executing a separated manufacturing process of an autologous therapy. A facility like this, connected to a digital twin for process monitoring and control, would make for a powerful manufacturing tool for autologous TEPs (and advanced therapies in general).

However, considering the complexity of certain tissues, let alone full organs, the current Cocoon is still only a first step on the long road toward full organ manufacturing.

While it enables a closed and automated process, it could be improved by adding a broader capacity for process parameter monitoring, linked to ML-based soft sensors and controls able to effectuate in-process adjustments. This, however, is limited by the current state of development of the soft-sensing and digital twin fields.

Regulators will also have to follow suit and embrace more flexible manufacturing techniques to accommodate patient-customized processes. A way to achieve it is through the implementation of the QbD methodology. This way, as long as the product critical quality attributes remain within specifications of the normal operating range, it should be safe and efficacious to be used in patients.

20.8 Recommended literature

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20.9 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
 1. Describe in layman's terms what a digital twin is and what it can be used for.
 2. Why is soft sensing essential for personalized TEP manufacturing?
 3. What is process robustness and why is it important?
 4. What is the connection between ATMPs and TEPs?
 5. Why is process scalability important?
 6. Mention two advantages and two disadvantages of both mechanistic and data-driven models.
 7. How can digital twins aid in reducing the cost and time needed to develop a TEP manufacturing process?
 8. What is the difference between an autologous and an allogeneic process or therapy?
 9. What is a Unit Operation? Give an example of a common Unit Operation in a TE manufacturing process.
 10. What is DoE? What is its role in Quality-by-Design?
 11. What is the role of Critical Quality Attributes in TE manufacturing?
 12. What is the role of Critical Quality Attributes from a regulatory standpoint?
 13. Why is process automation important?
 14. What is the relationship between Process Parameters, Material Attributes, and Critical Quality Attributes?
 15. What is the importance of a QC strategy?
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations.
 1. What is the added value of Quality-by-Design in the context of TE process development?
 2. For a selected TE publication, describe the manufacturing process of the TEP in terms of Critical Quality Attributes, Unit Operations, Process Parameters, and Material Attributes
 3. What are the main challenges in TE manufacturing?
 4. What is the importance of product quality?
 5. What are the unit operations and the process parameters of the Cocoon bioreactor?
 6. What is the added value of digital twins for process development?
 7. Research and describe an analytical method for one of the most common TEP CQAs that is nondestructive and automatable
 8. What is the potential of personalized medicine? What extra challenges does it bring?

Challenge-based learning

Industrial manufacturing of iPSC-derived tissue engineered products to treat macular degeneration

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

The last step in research and development of a tissue-engineered product (TEP), before it can be introduced in the market, is to set up an industrial manufacturing process. Manufacturing of TEPs follows a specific regulatory framework which focusses not on the efficacy of the product but rather on the technical requirements of the manufacturing process. The process needs to be described in meticulous detail. Because every successful research line in tissue engineering will eventually enter into this phase, our vision is that future tissue engineers should include the aspects of the product manufacturing process early in the exploration phase.

Motivation and stakeholders

Currently, there is a mismatch between the TEP production process in academic laboratories and manufacturing processes for clinical-grade products. Therefore, many TEP manufacturing processes are underdeveloped and suboptimal. In these cases, process scalability and reproducibility represent a major hurdle. To bring a TEP to market, engineers need to generate a detailed design of the manufacturing process. Modern process management strategies, including the quality-by-design concept, are increasingly used to set up manufacturing processes for TEPs in the clinic. Attempts to implement these strategies for TEP manufacturing should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as patients, surgeons, tissue and biomedical engineers, manufacturing engineers, chemical engineers, regulatory bodies, industrial engineers, and biotechnology engineers involved in the manufacturing of TEPs.

Problem definition

Macular degeneration is treated with iPSC-derived epithelial cells, but in order to be used as a TEP, the process has

to comply to the regulation of the Advanced Therapy Medicinal Products (ATMPs), Gene Therapy Medicinal Products (GTMPs), and Cell Therapy Medicinal Products (CTMPs). Hence, the process has to be described according to these regulations.

Challenge

To design an efficient and cost-effective TEP process following Quality by Design (QbD) methodology for the generation of a cell therapy medicinal product from iPSCs to treat and cure macular degeneration.

Learning framework

Reading the Product and Process Design chapter and related literature will help you to:

1. Understand the rules set out by the regulatory bodies on ATMPs.
2. Know the nomenclature of the process such as unit operations, process monitoring, and scalability.
3. Acknowledge the advantage of design requirements in the QbD methodology.
4. Comprehend the advantages of automation, single-use equipment, and improved mathematical process models involved in the manufacturing of ATMPs.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

5. The steps for current TEPs to comply with EMA regulations.
6. Cell culture systems used in TEP manufacturing.
7. Unit operations in the production of cells for macular degeneration treatment.
8. Aspects of the manufacturing process to be described according to the regulations.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

20.10 Glossary

Advanced therapy medicinal products are advanced therapies that are based on cell therapy, gene therapy, and engineered tissues, sometimes in combination with a medical device.

Critical quality attributes are physical, chemical, biological, or microbiological properties or characteristics of a product that should be within an appropriate limit, range, or distribution to ensure the desired product quality.

Data-driven model uses historical process data to build a mathematical model, such as cell counts, cell seeding densities, or nutrient consumption rates, to predict the evolution of a biological system.

Design-of-Experiment(s) is a statistical method to study the relationship between multiple input variables and key output variables.

Digital twins are a virtual representation that serves as the real-time digital counterpart of a physical object or process.

European Medicines Agency (EMA) is an agency of the European Union in charge of the evaluation and supervision of medicinal products.

Gene therapy medicinal products contain recombinant nucleic acid administered to human beings for therapeutic, prophylactic, or diagnostic effect.

Material Attributes are the properties of a material used in a manufacturing process, for example, the age of the cell donor or the glucose concentration in the growth medium.

Mechanistic models are predictions about a process based on the fundamental laws of natural sciences.

Personalized medicine is a medical model that separates people into different groups—with medical decisions, practices, interventions, and/or products being tailored to the individual patient based on their predicted response or risk of disease.

Pharmaceutical quality system ensures that the medicinal product or drug is of the quality required for its intended use.

Process Parameters are the measured values of various parts of a process which are being monitored or controlled.

Quality by design is an approach that aims to ensure product quality by employing statistical, analytical, and risk management methodology in the design, development, and manufacturing of medicines.

Quality control is a process by which regulatory entities review the quality of all factors involved in production.

Risk-based approach is an approach that allows identification of potential risks of a process and develops strategies to avoid or alleviate them.

Soft sensing is a method that uses a software mathematical model to predict difficult-to-measure response variables. For example, the prediction of cell growth based on nutrient consumption rates in a biochemical process.

Somatic-cell therapy medicinal products contain nongerminal cells or tissues that have been manipulated to change their biological characteristics to cure, diagnose, or prevent disease.

Tissue-engineered medicinal products same as tissue-engineered product.

Tissue-engineered product is a therapeutic product containing engineered cells or tissues, which is intended to regenerate, repair, or replace a human tissue.

Unit operation is a single step in a manufacturing process. A manufacturing or biological process is usually the combination of two or more unit operations.

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Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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Clinical translation

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21.1 Learning objectives

After reading this chapter you will be able to:

- Get acquainted with the basic principles of the clinical development of conventional drugs.
- Understand some typical challenges encountered with the clinical translation of tissue-engineered products.
- Learn about the documents required for implementation of a clinical trial.
- Discuss special points to consider in the clinical translation of tissue-engineered products.
- Get familiar with some regulatory aspects in the clinical development of medical devices and tissue-engineered products in the field of regenerative medicine.

Lost in Clinical Translation

Nature Medicine editors, 2004.

Whenever a new discovery is reported to the scientific world, they say first, 'It is probably not true'. Thereafter, when the truth of the new proposition has been demonstrated beyond question, they say, 'Yes, it may be true, but it is not important'. Finally, when sufficient time has elapsed to fully evidence its importance, they say 'Yes, certainly it is important, but it is no longer new.

M. de Montaigne, 1533–1592.

21.2 Introduction

Since the beginning of this century, the field of **regenerative medicine** and **tissue engineering** (TE) has been changing rapidly, with new scientific and technology developments, leading to paradigm shifts from classical in vitro three-dimensional culturing of cells to in vivo restoring of tissue function. Concomitantly, the path of clinical development and related directives and regulation has also evolved, resulting in the perception that regulatory requirements and statutes have become “moving targets” that may hamper progress in the field. Using new approaches and technology in humans implies providing answers to new and unexpected questions and clearing challenging regulatory and ethical hurdles. Historically, medical devices and cell and tissue products had fewer regulatory requirements than the established class of drugs/medicinal products, and these requirements mostly focused on safety.

However, new regulation about medical devices (MDR 2017/745/EU) brought EU legislation in line with technical advances and changes in medical science. It was also recognized that many tissue-engineered products, in particular cell-based combination products, should meet the requirements of a demonstration of efficacy that apply for conventional drugs. Therefore, new regulation was put in place. These products are now categorized as **Advanced Therapeutic Medicinal Products (ATMPs)** under regulation (EC) N° 1394/2007 in Europe and as Human Cells, Tissues, and Cellular and Tissue-Based Products (HCTPs) in the US Code of Federal Regulations, Title 21, Part 1271 (further referred as 21CFR1271).

To be classified as an ATMP in Europe, a given biological product must fulfill the legal definition of a medicinal product and in addition the definition for somatic cell therapy, gene therapy, or a tissue-engineered product.

In the United States, the provisions of 21CFR1271 apply to all HCTPs. As outlined in 21CFR1271.10, some HCTPs are also regulated under Section 351 of the Public Health Service Act and/or the Food Drug & Cosmetic (FD&C) Act. For these products, 21CFR312 also applies as for conventional small-molecule drugs; an Investigational New Drug (IND) exemption is required for human studies. Additional requirements for licensed products classified as biologics are set forth in 21CFR Subchapter F of Chapter I (Sections 600, 601, and 610).

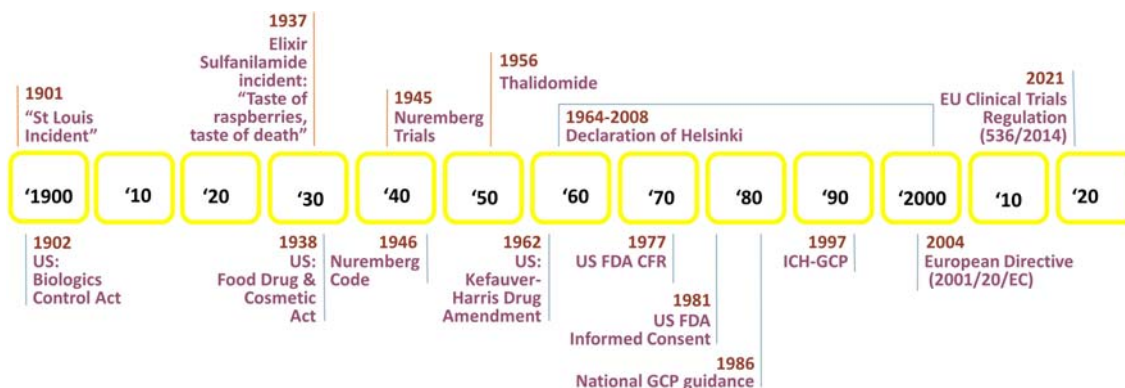
We will present an overview of issues and hurdles that may be encountered when developing tissue-engineered products in the field of Regenerative Medicine and will try to provide a framework on how to translate these innovative therapeutic regenerative approaches in a practical and safe way into daily clinical practice with respect to the perspective of the patient and worldwide standards of **Good Clinical Practice (GCP)** as outlined in the guideline of the International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use, ICH E6 (ICH E6, 1996). Though we will consider the new medical device regulation in Europe briefly, most attention will focus on the new ATMP regulation for cell-based products highly relevant to the field of regenerative medicine.

21.2.1 Historical perspective

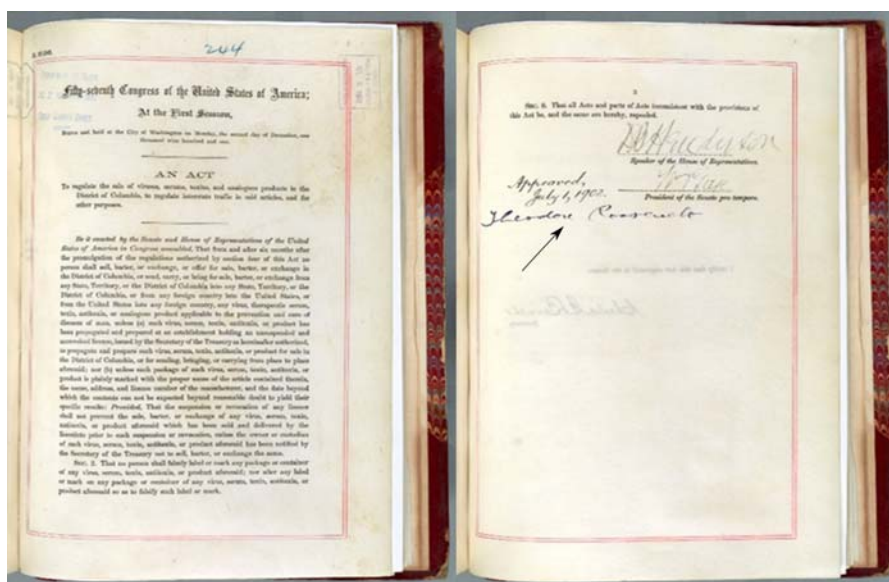
To better understand the development of regulation in the field of regenerative medicine and TE, it is important to understand how the execution of clinical trials changed over time.

Historically, the rules for performing clinical trials were developed and adapted because of incidents occurring with new treatments (Fig. 21.1).

One of the first documented medical disasters was the St Louis Incident¹ where young diphtheria patients were treated with an antitoxin, produced in a horse. At that time, the therapy was very successful and proven effective for over 6 years. The specific antitoxin used was contaminated with tetanus because the horse was infected. As a result, treated patients did not die from diphtheria but from a tetanus infection. This incident prompted Congress to pass the Biologics Control Act (1902) (Fig. 21.2). This act and following legislation resulted in creating the Food and Drug Administration (FDA), which aims to increase the safety and public confidence in lifesaving new therapies.

**FIGURE 21.1**

Historical events leading to today's regulatory framework.

**FIGURE 21.2**

Biologics Control Act (1902) signed by President Theodore Roosevelt (black arrow). Thanks to: Jane Fitzgerald, Archivist, Archives 1 Reference Section, National Archives and Records Administration, Washington, DC.

Another important event was the "Elixir Sulfanilamide" incident (1937),² which was called "taste of raspberries, taste of death." "Elixir Sulfanilamide" was a drug used to treat *Streptococcus* infection. Because these infections often affected children, a better-tasting syrup was developed for pediatric use: the sulfanilamide was dissolved in diethylene glycol and the juice of raspberries was added. Testing was done on the solution to evaluate taste and smell as was required at that time. Tragically, 107

people, most of them children, died because of the deadly ingredient diethylene glycol (commonly used as antifreeze, now known to induce often lethal central nervous system damage, nephrotoxicity, and metabolic acidosis). Because of this event, the FD&C Act (1938) was adopted and stated that data on safety had to be evaluated before approval by the FDA.

During the Second World War (1940–45), unethical medical experiments were performed by German doctors on concentration camp inmates and civilians of occupied countries. This horror resulted in the Nuremberg Code (1946), the first ethical code for experiments on humans with voluntary consent as its most important principle.^{3,4}

The Softenon (thalidomide) incident (1956)⁵ confronted scientists with the fact that, for new medicines, not only were safety and short-term effects important but also the long-term effects, including reproductive toxicity, had to be studied. Softenon was used as an effective treatment against morning sickness in pregnant women, but many severe birth defects were seen in babies of mothers who took the medicine. This event led to an amendment of the FD&C Act, better known as the Kefauver–Harris Drug Amendment (1962). This amendment expanded the requirements of the regulatory bodies (FDA) to oblige manufacturers not only to provide evidence of safety in the short and longer term but also to demonstrate efficacy before approval. This amendment essentially compelled the biomedical/regulatory community to develop the current paradigm for **staged clinical trials** to support marketing approval of medicinal products.⁶

In 1964, the World Medical Association adopted the “Declaration of Helsinki,”⁷ which stipulated the ethical principles for medical research involving human subjects.⁴ The basis of this declaration was the Nuremberg Code and this declaration led to the most important principle still used in clinical trials: Good Clinical Practice. GCP is the international ethical and scientific standard used in the design, execution, verification, analysis, and reporting of clinical research in the “human model.” This concept is to ensure that results generated by clinical trials are scientifically rigorous, reliable, and collected with respect to the integrity and privacy of the research subjects in such a way as to maximize generalizable knowledge beneficial to the human community while protecting the safety and rights of subjects.

In the beginning, several GCP guidelines were developed, resulting in country-specific interpretations of this universal concept. Fortunately, in 1997, an international conference was organized to harmonize the guidelines between the United States, Europe, and Japan. Therefore, clinical trials conducted according to the International Council for Harmonization (ICH) E6 GCP guidance can be submitted in support of Marketing Authorizations (MkAs) in all regions of the ICH (ICH E6, 1996).

Since then, the ICH guidelines, including ICH-GCP, have been referred to in legislations worldwide. For Europe, these guidelines are part of the European **clinical directive** (2001/20/EC). A directive sets a legislative goal for European member states that needs to be translated into National law, whereas a **regulation** is binding in its exact wording for the entire EU. The implementation of the clinical trials directive into national laws gave rise to a degree of variability between distinct European member states and resulting difficulties for applicants conducting multinational trials. For example, in Belgium, no distinction is made between clinical research with registered medicines and studies involving new medicines, while this distinction is made in other member states, as, for example, in the Netherlands. Consequently, investigator-initiated research with approved medication must be registered, under Belgian Law, centrally as European Union Drug Regulating Authorities Clinical Trials (EudraCT) in

Belgium, but not in the Netherlands. To ensure harmonization and to remove obstacles for clinical researchers, the directive (2001/20/EC) was replaced by the EU Clinical Trial Regulation (536/2014), implemented on January 31st 2022, and executable in full from January 31st 2023. With a grace period till January 31st 2025 for trials started under the directive.

For the US, ICH-GCP guidelines were published as a guidance document in the Federal Register in May 1997 and are not legally binding. Parts of the guidelines were adopted as regulations and are legally binding, i.e., ICH E2 on Clinical Safety Data Management. On other points, there are differences between the ICH guidelines and FDA requirements. For example, regarding responsibilities of the Institutional Review Board (IRB, also called independent Ethics Committee) (ICH 3.1), where FDA is stricter, and investigator requirements (ICH 4) for which ICH is more demanding.⁸

21.2.2 Framework for clinical development of conventional medicinal products

As mentioned above, clinical research involving drug development is strictly regulated and a brief overview below can serve as a reference for the development of tissue-engineered products. It is divided into different phases; typically, over a decade of research and development is needed to get a Central Market Approval.

In contrast to TE products, conventional drug discovery programs typically start with large-scale screening using a variety of methods. Nonclinical research usually involves further in vitro testing in cell and tissue cultures and in vivo experiments in relevant animal models if appropriate.

Clinical drug development in humans is divided into phases I–IV, each with its specific focus as outlined in ICH E8 (ICH E8, 1997) and 21CFR312.22 (Table 21.1) and described below.

In conventional models of drug development, the first phase (**Phase I, human pharmacology**) involves small numbers of volunteers/patients and its objectives include initial assessment of safety and

Table 21.1 Different phases of clinical drug development.

Phase	Description
Phase I	Human pharmacology (PK/PD) Small number of volunteers/patients Safety Tolerance
Phase II	Therapeutic exploratory Small numbers of patients First signs of efficacy Dosing Safety
Phase III	Therapeutic confirmation Larger numbers Efficacy Safety
Phase IV*	Postmarketing evaluations
*Phase IV seeks Market Approval (MA)	

tolerance, evaluation of pharmacokinetics (PK) and pharmacodynamics (PD), and an exploration of drug disposition (disposition refers to what happens to the drug after it enters the body, including distribution and elimination processes) and interactions, with a first assessment of activity if appropriate. Over the most recent years, there has been a shift to using patients instead of healthy volunteers in this phase of drug development as the predominant concern of this phase is the prospect of benefit versus risk. For example, some cytotoxic chemotherapeutics would put a healthy volunteer to an unreasonable and significant risk of illness or injury (CFR 312.42(b) (1)). This suggests that the physician responsible for treating the patient needs to be closely involved, so this benefit risk ratio can be always monitored in comparison to standard care and disease management. Risks and the availability of alternatives should be discussed during the **informed consent** process. Early on, it is important to determine the highest safe dose of the product with carefully chosen dose escalation studies in small cohorts, typically starting with a single-dose step-up design followed by a multiple-dose scheme to determine the disposition of the drug. Dosing studies are quite a challenge in the case of cell-based ATMPs, as increasing dose does not automatically imply enhanced therapeutic effects, and even the definition of “dose” can be tricky (see [Section 21.3.2](#)).

In the second phase (**Phase II, therapeutic exploratory**), the use for the targeted indication is explored for efficacy and more detailed investigations into dosage regimens for subsequent studies conducted in small groups of patients. The phase II studies provide additional safety data, evaluate the first signs of proper dosing and efficacy in some cases (depending on treatment effect size and sample statistical power), and explore other product- or indication-specific trial design elements to help form a solid basis for the optimal confirmatory study design, including endpoints and methodologies.

The last phase (**Phase III, therapeutic confirmatory**) is called pivotal and aims to provide efficacy and safety data for assessing the benefit/risk in a sufficiently large group of patients to support licensing. Therefore, efficacy needs to be demonstrated in this phase and the safety profile should be evaluated in a sample large enough to provide sufficient **statistical power** to detect relatively rare events. This involves in general at least two independent studies, in which the second study is anticipated to confirm the results of the first pivotal trial. In some cases, a single trial may suffice, if adequately designed and controlled.

In the context of ATMPs, deviations from this “classic” phasic model are seen with adaptive protocol designs and shifts from healthy volunteers to patients, as described earlier.

Some medical therapies may offer treatment effects so large that definitive efficacy data may be obtained earlier than expected. In these cases, the size of the safety database (DB) required depends on the gravity of the indication; treating baldness is not the same as treating Parkinson’s.

New medicinal products are required to provide sufficient efficacy data to support marketing approval. Under the prerequisite that efficacy has been demonstrated, EMA and FDA provide a path for a conditional **Market Approval** (MA) or an accelerated track with the requirement of providing data on the efficacy and safety within a specified time with postmarketing commitments where appropriate.

Most clinical trials in the first 3 phases of clinical research are conducted by commercial sponsors seeking MA. **Phase IV studies**, in contrast, are conducted by academic and commercial sponsors on approved medicinal products. The distinction between phase IV studies and noninterventional studies is that in the latter no interventions beyond routine practice are permitted, that treatment needs to be done according to the conditions of the marketing authorization, and that patients can only be

included after the treatment decision has been taken. Randomization and additional diagnostic tests, for example, therefore belong to a phase IV setting. Phase IV trials are setup to answer questions not included in the process of MA, for example, on disease management, cost effectiveness, and long-term safety in real-life clinical practice. The latter can be requested by the competent authorities.

Considerations other than MA, although outside the scope of this chapter, may be important in health policy decision-making. **Health Technology Assessment (HTA)** is intended to support policymakers with information on how to provide safe, effective, patient-centered, and cost-effective treatments. In this context, effectiveness in comparison with existing standard of care might be informative. In a recent EU initiative, Eunetha has been created to facilitate harmonization between national assessment bodies (see **Recommended website 1**) and EMA provides the option of joint EMA/HTA scientific advice (see **Recommended website 2**). Moreover, it may be useful in certain cases to design a single trial to provide information sufficient not only for MA but to guide or inform intelligent decisions regarding reimbursement.

21.3 Clinical translation of tissue-engineered products

Translating the regulations developed for classical medicinal products to medical devices and tissue engineered products, for regenerative medicine applications, may often appear challenging in this young and quickly developing field. However, interactions with both FDA and EMA reveal a high degree of flexibility for the investigational phase of new and innovating TE products.

We discuss development and the main hurdles of medical devices in view of the new European regulation and present the framework for the development of products that can be categorized within the ATMPs/HCTPs with relevance to the field of TE and regenerative medicine.

21.3.1 Medical device regulation

A tissue-engineered product can be designated as a **medical device** or an ATMP, based on its composition. Typical examples of devices in the field of TE are biological or (semi)synthetic membranes or matrices intended for wound healing.

In general, for implantable medical devices, the **technical product documentation**, a **quality management system**, and if appropriate an **active substance file** should be submitted to the **Notified Bodies** designated under the new Regulation. A preclinical track should be developed and refined to make sure materials used were tested sufficiently, primarily for safety/toxicity before clinical use. For regenerative medicine applications, one should test the device in vitro as well as in vivo, to investigate how the device integrates in living tissue or how it changes the structure and/or the biology, behavior, and remodeling of the surrounding tissue. A new Medical Device Regulation (MDR) was released in Europe in 2017 (2017/745/EU). This MDR replaces the existing medical devices Directive (93/42/EEC). In contrast to a directive, a regulation does not need to be transposed into national law, thereby avoiding discrepancies in interpretation across the EU market. There was a transitional period that ended May 26, 2021, the Date of Application of the regulation. An additional grace period of 4 years is foreseen for some devices with certificates issued under the directives; these devices will be made available until May 26, 2025.

In the field of TE, engineered implantable devices such as optimized synthetic membranes or matrices with regenerative potential intended to enhance tissue repair are quite typical products. For instance,

bio ceramics to enhance endogenous bone formation for the repair of bone defects are currently considered as Class III medical devices. In Europe, the new MDR sets rules for determining the risk classes (Annex VII; Class I, IIa, IIb, and III) with special attention to implantable devices. There are different routes of assessment according to the class of the device. The MDR adds new requirements, placing more emphasis on a life-cycle approach to safety backed up by clinical data. Indeed, for certain Class IIb and III devices, there is a new clinical evaluation consultation procedure to be carried out by an independent expert panel. Importantly, the scope of the Quality Management System now also includes postmarketing clinical follow-up. Moreover, there will be a new European Database for Medical Devices EUDAMED, which includes the unique device identifier DB, playing a central role in making data publicly available and increasing the quantity and quality of the data. This clearly heightens the bar for market approval, particularly for the field of regenerative medicine and TE, but these changes will likely be beneficial for the patient and society. Guidance for the evolving new regulation is provided by the Medical Device Coordination Group, with members of the European Commission and Member State representatives.

As a rule of thumb, adding gene therapy and/or (engineered) living cells to an implantable device moves the product development into the ATMP track, a distinct new class of medicinal products with its own regulatory track in Europe.

21.3.2 Advanced therapy medicinal products

A novel class of products can be designated as advanced therapy medicinal products (ATMP) in Europe or HCTPs in the United States (21CFR1271, 2005), the typical active substances being engineered/manipulated cells and/or tissues. A list of approved ATMPs in Europe and in the United States of America with latest updates can be found on Recommended websites 3 and 4.

Additional substances (scaffolds, matrices, devices ...) can be combined with the ATMPs/HCTPs. The definition of a "Combined advanced therapy medicinal product" requires that the ATMP/HCTP incorporates, as an integral part, one or more medical devices that act in their primary action on the human body. Most of the ATMPs/HCTPs used in TE contain cells of human origin. Even so, these products are heterogeneous regarding the origin (autologous or allogeneic), type of cells (e.g., adult mature cells, adult progenitors, stem cells), and in the complexity of the product. Standardization of the process therefore needs to include the procurement step to ensure that the starting material for manufacture is consistent. Further, the development of this type of ATMPs/HCTPs requires in depth characterization of the product/cells intended for the treatment and to ensure that the final product can be manufactured robustly and reproducibly (Table 21.2). A facility with documented proof of adherence to GMP (good manufacturing practice) is essential for a standardized manufacturing process. Consistent manufacture is vital, but particularly challenging for tissue engineered ATMPs/HCTPs, some of which are patient tailored. As an example, in the case of Autologous Chondrocyte Implantation (ACI), chondrocytes obtained from an articular cartilage biopsy of the patient are used to repair symptomatic joint surface defects in the same patient. The number of chondrocytes needed depends on the size of the lesion. Thus, it is important to have a certified GMP facility that can provide a product containing well-characterized viable chondrocytes in the required amount.

Importantly, before investigating and introducing a specific tissue-engineered product, one should define the patient and identify the unmet medical need. This includes a critical evaluation of all

Table 21.2 Characterization and product specifications.

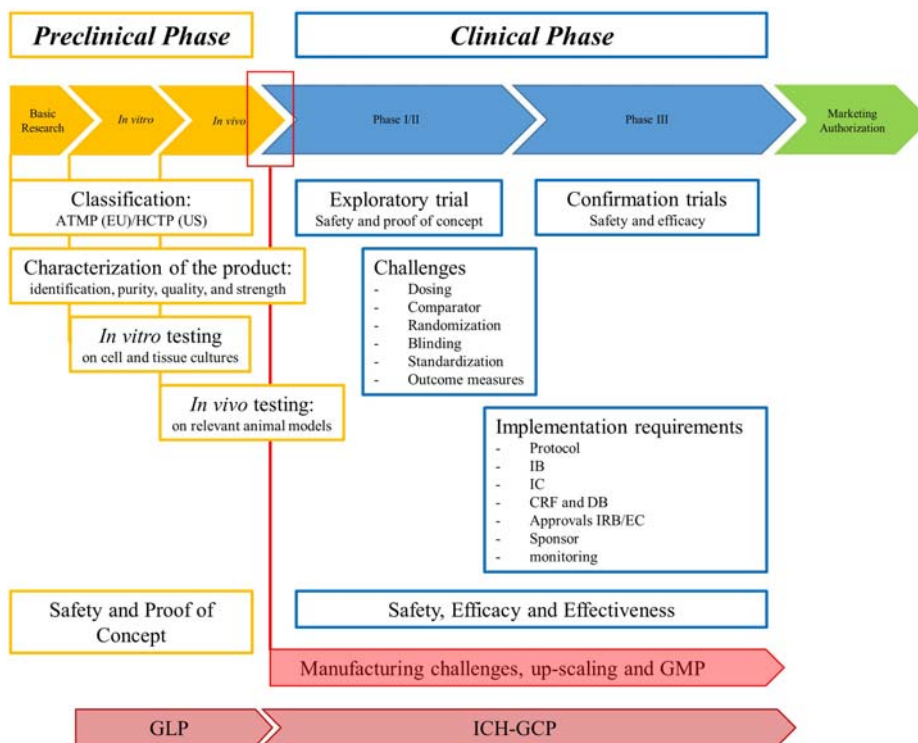
Test	Method	GMP goal
Identity	Morphology Biochemical markers	Quality Safety
Dose	Viable cell number	Quality Efficacy
Potency (strength)	Relevant bioassay potentially replaced by marker profile	Efficacy
Purity	% Cell viability Residual impurities	Safety Efficacy
Safety	Sterility Mycoplasma Endotoxins Adventitious pathogens	Safety

available and relevant scientific literature to validate and refine the proposed treatment concept/product, to ensure quality, safety, and efficacy. When the patient and treatment are well defined, a preclinical path can be designed. In case of doubt, for Europe, Regulation 1394/2007 amended Directive 2001/83/EC and Regulation 726/2004 provide an optional procedure to determine whether a product/medicine can/will be classified as an ATMP. This application is handled by the Committee for Advanced Therapies (CAT) which provides an opinion to EMA within 60 days. In the United States of America, for FDA, the request for Regenerative Medicine Advanced Therapy (RMAT) designation must be made either concurrently with the submission of an IND application or as an amendment to an existing IND. This designation can be obtained if:

- The drug is a regenerative medicine therapy, which is defined as a cell therapy, therapeutic TE product, human cell and tissue product, or any combination product using such therapies or products with some exceptions.
- The drug is intended to treat, modify, reverse, or cure a serious or life-threatening disease or condition.
- Preliminary clinical evidence indicates that the drug has the potential to address unmet medical needs for such disease or conditions.

The development path of an ATMP is represented schematically in Fig. 21.3. Overall, it is not that different from the more conventional drug development path, containing both a preclinical and clinical phase. Therefore, it is preferred to keep this phasic scheme rather than a **Technology Readiness Level** (TRL) scheme, typically used in Europe for medical devices (like a prosthesis) alone. If wanted, the pre-clinical phase could be categorized from TRL1-3 where the clinical phase could be seen as TRL4-8 and the marketing as TRL9.

However, there are several ATMP-specific aspects discussed in more detail below.

**FIGURE 21.3**

Schematic overview on the preclinical and clinical development path of tissue engineered products.

21.3.3 ATMP preclinical phase

Before entering the human model, preclinical data should be obtained to provide a therapeutic rationale and determine ethical justification for putting human subjects at risk. Characterization of the product involves testing the widest variety of potential **Quality Attributes** that is feasible. Sufficient information must be submitted to ensure proper identification of the product, purity, quality, and strength (21CFR312.23(a) (7)). Although challenging, the development of a suitable **potency assay** will be needed prior to pivotal trials and should therefore be considered early on in development. Potency is defined as the quantitative measure of biological activity based on the attributes of the product, which is linked to the relevant biological properties. The assay(s) should be based on the intended biological effect(s) and ideally be related to the clinical response. As in the development of medicinal products, in vitro assessments including cell and tissue cultures are used most. Alternatively, in vivo models can be proposed, but are more challenging. As an example, the use of a cell suspension for cartilage repair requires the injected cells to make a hyaline-like matrix via mechanisms of action like engraftment and matrix deposition. As pointed out above, this is necessary to characterize the ATMP and to assess whether the product can perform its intended functions with an acceptable safety profile. This last aspect is frequently quite challenging, as in many cases the knowledge of the mode of action, though extremely useful, is limited. Indeed, predicting the cause of the therapeutic effect remains one of the most challenging aspects of cell-based therapies; an example is described in a classical experiment. Gaining in-depth understanding of the **mode of action** during this phase of research is desirable

for further development and optimization of the product, and in extenso the manufacturing process of the ATMP/HCTP. As an example, the use of autologous chondrocytes (Autologous Chondrocyte Implantation-ACI) to repair symptomatic joint surface cartilage defects seems quite straightforward, as the implanted cells, based on cell tracking experiments in appropriate animal models, appear to contribute directly to the repair process. More challenging is the injection of expanded adult bone marrow stromal cells into osteoarthritic joints, as the primary mode of action, if any, is not known. Since there is no indication of cell engraftment, a paracrine function seems possible, although it is difficult to identify which paracrine factors are critical for the hoped-for clinical outcome, i.e., symptomatic improvement and repair of structural damage.

Following in vitro assessments, in vivo studies may be required. Clinically relevant animal models have to be identified and applied. Mice and rats can be used to generate data on both “behavior” and safety of the product, but confirmatory studies in a relevant in vivo environment may be needed to provide adequate therapeutic rationale to justify human experimentation. Depending on the clinical problem and/or patient context and considerations of **allometric scaling** (a prediction method to provide a “sneak peek” at how a product might behave in humans before any clinical studies are conducted), relevant physiology, or biomechanics, this is often done in a larger animal model, ideally one that resembles the intended clinical situation closely. In the example of ACI, mice and rats cannot mimic the condition of interest, owing to both allometric scaling and **biomechanical limitations**. In the case of ACI, the use of a large animal model is appropriate. For example, the goat, sheep, or the horse model may offer much more informative assessments, as the size of the joints and the thickness of the articular cartilage are quite relevant when compared to the human joint (recommendations provided in Recommended website 7). Biological findings that are conserved across species provide confidence in the behavior of the product. Gathering nonclinical data is a time-consuming undertaking, not only due to the need for different in vitro and in vivo models, but also for follow-up that is sufficient to ensure the safety of the product and durability of the therapeutic effect. For ACI (for example), this would imply a minimum of 2 years follow-up, since the clinical data indicate that the 2-year results predict the longer term 5-year and 10-year data.

Preclinical studies commonly focus on proof-of-concept, such as, for instance, regeneration of a joint surface defect. However, safety concerns including the **biodistribution**, i.e., whether and in what quantities cells will migrate through the body to unintended sites, tissues, and organs including the gonads, as well as other aspects of pharmacological disposition, are also crucial. Another consideration is that the ATMP/HCTP used in a preclinical setting may not always equal the final cell product used in humans. For example, human cells used in animal models can trigger immunological reactions with the rejection of the donor cells. Thus, in some cases, testing may better be done with homologous cells from the same species to clearly identify the mode of action rather than using the human cells in an immunologically impaired animal model. Ideally, one seeks to obtain similar data with both the homologous and the “real” product, or at least identify the similarities and differences and their potential impact in the human model.

21.3.4 ATMP clinical phase

The clinical phase in TE does not simply copy the expertise and experience of the different phases from the development of conventional drugs. To set up a clinical program for ATMPs/HCTPs it is highly recommended to contact the regulatory agencies (CAT/EMA and/or FDA) early in the process to discuss

the program. Both agencies have specific mechanisms to aid in the designation of a product as medical device, ATMP/HCTP, or combination product. This designation can be obtained informally early in the development process and is at this stage not legally binding. Once a designation is formally requested and obtained then it becomes binding. For EMA, this is the Committee for Advanced Therapies (CAT), which also provides a tailored certification procedure for ATMPs. In the United States of America, Requests for Designation are considered by the FDA's Tissue Reference Group, consisting of representatives from the Center for Biologics Evaluation and Research (CBER) and the Center for Devices and Radiological Health and, where applicable, the Office of Combination Products. The review center and office identified by this process will provide the primary contact for advice.

Both EMA and FDA are involved in centralized licensing procedures. At the end of the route, in Europe the CAT will present a report and an opinion to the Committee for Medicinal Products for Human use (CHMP). The CHMP will finalize the procedure and send the final opinion to the Commission, to the member states, and to the applicant. The Commission will then take a final decision on the procedure.⁹ In the United States, if classified as a Biological License Application, the OTAT (Office of Tissues and Advanced Therapies) of CBER will evaluate the license application and either approve it or provide a complete response to the sponsor. The basis of the evaluation to grant an MA by the regulatory agencies for an ATMP/HCTP is done using a risk-based approach based on the totality of data on the product and the indication.¹⁰

Since 2004, FDA and EMA have implemented a dialogue on the development of ATMPs/HCTPs known as the Parallel Scientific Advice (PSA). This forum facilitates harmonization of the views of the different stakeholders, allowing them to avoid duplication of efforts in the development of ATMPs/HCTPs and provide consistent advice to regulated industry. The idea of the PSA became more formalized in 2008 when FDA and EMA established the US FDA-EMA ATMP cluster, which was joined by Health Canada in 2012.¹⁰

Thus, first steps toward harmonization have been taken. However, FDA and EMA currently can provide different advice or have different requirements. Therefore, the ICH has organized a Regulatory Forum on cellular therapy intended to create an environment and roadmap to enhance the availability of safe and efficacious ATMPs/HCTPs worldwide. More recently, regulation in Japan has also undergone substantial changes, but a more detailed comparison and discussion is outside the scope of this chapter.

21.4 Typical challenges for tissue engineering encountered in the clinical phase

As described above, it's not possible to copy/paste the process for the development of conventional medicinal products into development of an ATMP/HCTP. These challenges have been recognized and illustrated by a recent guidance of an Advisory Committee in the United States of America discussing on the topic of trial design for cell therapies. Unfortunately, randomized controlled trials (RCTs), which typically seek to repair or regenerate damaged tissues such as the skin, cartilage, bone, intervertebral discs, myocardial tissue, or peripheral nerves and tendons, are not common, and quite challenging to execute in the field of TE. Thus, only few examples exist. However, RCTs remain the only accepted

standard for proving efficacy and safety. An example of such an RCT is given in the State-of-the-Art Experiment (separate box).

In the next paragraphs, an overview of several, but not all, challenging issues will be covered that need attention in designing and implementing such surgical trials.

21.4.1 Exploratory trial

The concept of phase 1 research on healthy volunteers, as is standard for conventional drugs, is not appropriate for most ATMPs and often will not add adequate information. Most guidelines from regulatory agencies will allow an exploratory trial establishing safety and proof of concept as a starting point. This involves a small group of patients, sequentially escalating doses, and looking for biological effects that reflect to some extent the results in the animal models. It further evaluates the feasibility of the treatment in the human model and aims to detect catastrophic safety issues that could not be predicted in animal studies or other unexpected findings.

21.4.2 Dosing

Conventional notions of dose do not always apply for tissue-engineered products. To offset unavoidable risk to some extent, it may be appropriate in many cases to choose initial doses that offer a realistic possibility of therapeutic benefit. This is a substantial departure from the logic of conventional small-molecule trials. Moreover, a maximum dose is limited not only by potential toxicity, but by other determinants including the size of the site of administration, the number of cells that can be produced, or other factors defining a 'maximum feasible' as opposed to 'maximum tolerated' dose.

21.4.3 Defining the comparator

As the trials in TE frequently involve surgical procedures, one of the first hurdles to clear is to define the **comparator**, i.e., the currently accepted standard treatment for the specific clinical problem to be investigated. The lack of a generally accepted comparator or control group is common, as are differences in treatment approaches across countries and continents. Choosing the standard of care in daily practice as a comparator can raise the demand for supplementary research by the regulatory agencies because the standard treatment is not necessarily evidence based. The standard of care should be proven superior to placebo where possible, but in several cases this was/has not been done, is or was not feasible, or ethically was not defensible. The use of coronary artery bypass in patients was widespread prior to a careful analysis of outcomes and a particularly compelling case in point of the importance to carefully investigate proper controls.¹¹ Another example relates to articular cartilage repair, where the technique of Micro-Fracture (MF) was selected as comparator. In this technique, the surgeon drills holes in the subchondral bone to create repair tissue coming from the underlying bone marrow that will fill the cartilage defect. Subsequently, it turned out no one had proven that this therapy/technique was superior to placebo or a conservative approach without surgical intervention, and the regulatory bodies demanded proof of superiority. Therefore, the effectiveness of MF had to be evaluated in addition to the proposed experimental therapy. An extra burden on the control groups in ATMP/HCTP RCTs is that in most cases the (surgical) techniques to apply the ATMP/HCTP are more demanding than in classical medicinal products (pills, infusions, or subcutaneous injections) and bear risks on their own (Table 21.2).

In addition, a device like a specific joint prosthesis is not always the most sophisticated on the market but in the hands of an experienced surgeon the results using that prosthesis can be excellent. Therefore, the “best” device cannot guarantee the best efficacy when applied by someone who is not trained for and experienced with the specific device and associated technical skills. This brings us to a next point of attention: the complexity of the procedure to apply the ATMP/HCTP can influence the efficacy of the ATMP/HCTP and certainly change the relevant timelines for assessment. An example of a more complex procedure compared to MF is ACI for a joint surface lesion. This procedure starts with debriding and measuring the lesion, followed by cutting a collagen membrane to the appropriate size and seeding the chondrocytes. The final step in this procedure is the application and suturing of the cell seeded membrane to the lesion (Fig. 21.4). All steps should be mastered by the surgeon to obtain a satisfactory result. So before starting a clinical trial, it may be important to train the investigators in all procedures when applying the ATMP/HCTP. One must be aware that most techniques require a learning curve, and so a standardization program should be in place to make sure the registered efficacy results from the difference in treatment between groups and not the technical skill with which the ATMP/HCTP and control interventions are applied. To standardize this within a trial, a few options are available, ranging from training the procedures on animal models to appointing a single surgeon who will be applying the ATMP/HCTP in all patients.

In view of this, it is critical to consult all partners involved, including the key opinion leaders and regulators, to come to a broadly agreed comparator group, and the appropriate technique to “deliver” the ATMP/HCTP to the patient.

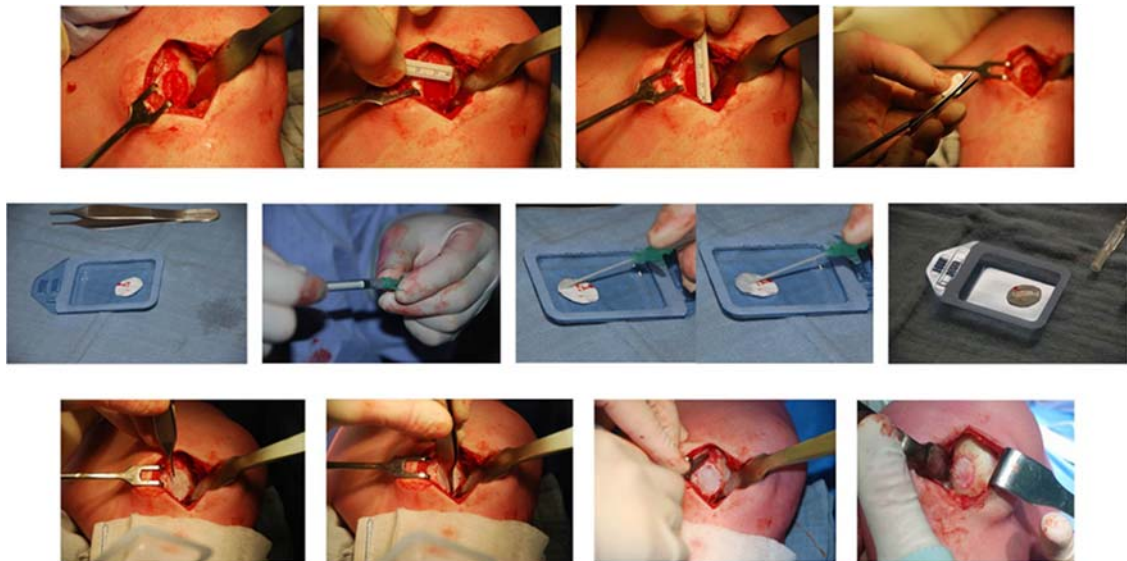


FIGURE 21.4

Example of the complexity of an ATMP/HCTP administration.

21.4.4 Randomization

Randomization in clinical trials is a tool to minimize differences between the groups to be studied at the start of the trial, typically the treatment group and the comparator. Thus, every group should have a similar distribution of relevant predefined demographic data and disease parameters. Moreover, randomization also implies that every participant has the same chance of receiving the treatment studied. Randomization procedures are standardized and independent of the investigator. Most of the time, a computerized system is used for the assignment of patients to groups. For ATMPs/HCTPs, this already implies a hurdle: how to organize this randomization procedure when decisions are made in the operating theater? A possible solution would be to call in to a central phone number that blindly assigns the patient to a treatment arm.

21.4.5 Blinding a trial

Double-blind design in RCTs is intended to minimize the potential for placebo effects. When both the patient and treating physician (investigator) are unaware of the treatment assignment, all decisions are much more likely to be based on the clinical presentation and unaffected by knowledge of the treatment/procedure given.

In the field of TE this is not an easy task, if possible, at all. Most ATMPs/HCTPs are applied by specific (surgical) procedures, not allowing for a full **double-blind process**. In addition, any surgical intervention has a substantial placebo effect which can persist for 1–2 years. However, sham operated controls are not always acceptable ethically but may be justified scientifically; in such cases this point needs to be discussed with the key opinion leaders in the field, the regulatory bodies, the patients, and ethical committees.

Furthermore, the idea of double-blind design is to ensure that evaluation of the patients is done on clinical presentation and not biased by knowledge of whether a patient received the experimental ATMP/HCTP or the control treatment. Therefore, strategies for blinded evaluation must be implemented for patients in an RCT with these types of ATMPs/HCTPs where possible. The investigator who will make the assessments essential for the evaluation of the ATMP/HCTP has to be independent. In practice, the evaluation should be done by a clinician not involved in the initial treatment. Some of these techniques for independent evaluation can be adopted from experience in other fields, as, for example, from drug trials in rheumatoid arthritis, where joint counts are examined by rheumatologists not involved in the clinical decision-making of the trial.

21.4.6 Standardization of patient care and follow-up

The standardization of postintervention patient care and rehabilitation is crucial not only to optimize efficacy and safety but also to improve the recovery from sometimes complex procedures such as orthopedic interventions.

When a surgical technique is applied, an appropriate standardized wound care and rehabilitation plan is required, whether by delegating one person to follow-up on all patients treated within the RCT or by assuring that all care givers participating in the follow-up are trained appropriately. Measures to educate and train physical therapists, provide a consensus on standardized identical rehabilitation programs,

**FIGURE 21.5**

Physiotherapy as part of a standardized postoperative rehabilitation program is required to optimize wound care and rehabilitation after surgery.

stipulate an implementation plan, define criteria to measure progress in rehabilitation over a wide postoperative time, monitor patients' activities both in rehabilitation and during activities of daily living, and, if possible, organize supervised education and training (Fig. 21.5) should be stated explicitly.

In the case of ACI, the ATMP/HCTP is typically applied with a two-step surgical procedure: harvesting chondrocytes arthroscopically from the patient and 4–6 weeks later implanting the cell product with an open knee surgery in the same patient. In contrast, in the control group MF is done in a single procedure. The postoperative rehabilitation following an ACI procedure may result in another approach than the one used in the control group. Accordingly, the trial should be designed to minimize the influence of the procedure on postoperative management.

21.4.7 Outcome measures

The same caution should be applied for the primary and secondary outcome measures or **endpoints**. What seem to be “hard” outcomes in a clinical trial with medicinal products may not be so clear in TE. For example, pain is an outcome typically used in trials with medicinal products in musculoskeletal diseases such as rheumatoid arthritis, and it is validated for these diseases. For TE, this outcome would not necessarily measure what is intended. When a patient is treated with ACI and the appropriate open knee procedure is required, pain will be less relevant immediately after the procedure for evaluation of the efficacy, and only become relevant several months after surgery. Other “hard” structural outcomes, validated for development of medicinal products, may be requested by the regulatory agencies in RCTs for ATMPs/HCTPs. However, often they are not validated for tissue-engineered products and no standards or validated tools are available. This may mean that simultaneously with the trial, or even before starting the trial, suitable clinical or surrogate endpoints will have to be defined, evaluated, and validated. These endpoints should reflect the long-term efficacy within a reasonable follow-up period. Results of clinical trials reflect group-average effects which hold no guarantees for the outcomes for the individual patient in daily clinical practice.

21.5 Implementation of a clinical trial

21.5.1 Protocol

The process of developing a clinical trial starts with the development of the protocol. Content requirements for the protocol are outlined at the highest level in ICH E6 (ICH E6, 1996) (Table 21.3), with further information at the level of clinical trial guidance documents and guidelines. As described above,

Table 21.3 Content of protocol as described in ICH E6.**Clinical trial protocol and protocol amendment(s)**

General information
Background information
Trial objectives and purpose
Trial design
Selection and withdrawal of subjects
Treatment of subjects
Assessment of efficacy
Assessment of safety
Statistics
Direct access to source data/documents
Quality control and quality assurance
Ethics
Data handling and record keeping
Financing and insurance
Publication policy
Supplements

it is recommended to contact the regulatory agencies to discuss the protocol as soon as possible in the process of development.

The **protocol** is the key document that contains all information on the conduct of the specific trial. It is a blueprint of the complete process of what is going to be done, why it is to be done, how it must be done, and how it will be evaluated. The protocol starts with a general part on the rationale and introduction, the study design, the objectives, and the flow chart. In the next part of the protocol all items are discussed in more detail. The definition of the study population with inclusion and exclusion criteria, the primary and secondary outcome measures, the study duration, the enrollment period, the number of investigative sites, the clinical evaluations required per visit, stopping rules, informed consent procedure, randomization, statistical evaluation, laboratory tests, and technical investigations will need to be described. In the final part of the protocol more general issues will be addressed in more detail, including items such as how to handle safety reporting and how the safety committee is organized, the obligations of the investigator, compliance with the protocol and GCP, the Declaration of Helsinki, IRB/EC, informed consent process, data acquisition, source documents, retention of documents, confidentiality, deviations from the protocol and how to handle them, monitoring plan, final report of the trial results and subsequent publication policy, proposed analysis plan, and study material. The final version of the protocol must be signed on the protocol signature page by the sponsor and principal investigator (PI) confirming adherence to the protocol. The protocol is also referred to within the clinical trial agreement.

21.5.2 Investigator brochure

The investigator brochure (IB) is the document where information on the ATMP/HCTP relevant to the research program or on further products which are relevant to the study can be found. It is intended to

facilitate investigators' understanding of the product and contains information to support the rationale for dosing and scheduling schemes. The available data start with the nonclinical studies and pharmacology in vitro, followed by the PK, studies on product distribution, and behavior in animals as appropriate. The research on toxicology, safety, and geno-toxicity is also handled in the IB. Following the nonclinical data, conventional clinical studies in humans on PD and PK may be less relevant for cell-based products. On the contrary, dosage, rationale for dosage chosen, safety, and therapeutic rationale must be outlined. Because of the above, the IB is a dynamic document that needs to be updated during development as new information on the ATMP/HCTP becomes available. These updates need to be discussed with all participants in the clinical program and evaluated for their potential implications regarding safety and efficacy.

21.5.3 Investigational medicinal product dossier and investigational new drug application

The Investigational Medicinal Product Dossier (IMPD) in Europe is the document that captures the information on the manufacture and control of a given investigational medicinal product during the clinical trial stage and includes the technical information on the product. The IMPD is updated continuously as experience is gained with manufacturing and reflects the summary of the current and all historic manufacturing processes of the ATMP/HCTP.

In the United States, a medicinal product in development is called an IND and the documentation that needs to be submitted in support of clinical trials equally needs to capture extended information on the source, characterization, manufacture, formulation, stability, storage, and handling of the product. The explicit requirements are presented in the United States Code of Federal Regulations, Title 21, Section 312.23(a) (7) (21CFR312.(a) (7)). This section is called the Chemistry, Manufacturing, and Controls (CMC) part of the IND.

On both continents, detailed information is required on how the quality of the ATMP is guaranteed using GMP compliant facilities. The information contained in the IMPD/IND CMC forms the core of module 3 (Quality) of the Common Technical Document format for an MA.

21.5.4 Informed consent

Subjects need to consent to the study in general and to all related procedures, as well as understand their rights as participants, before any study related procedure is performed. To comply, subjects and investigator must sign the informed consent form in duplicate, one copy for the patient and the other for the investigator file to be kept at the site. The procedure must be clearly documented in the subject medical file.

The informed consent contains an explanation of the disease studied, the aim of the study, the study procedures, possible side effects of the procedures/ATMP/HCTP, information regarding reimbursement (if any) or insurance, patient rights, privacy, IRB/Ethics committee (EC) review, study related costs, data handling, General Data Protection Regulations, and alternative procedures including the right to withdraw or refuse without prejudice to their medical care. The content of the consent form can be found in ICH-GCP guidelines (see **Recommended website 8**).

21.5.5 Case report form and database

Before starting an RCT, a case report form (CRF) and DB must be constructed to collect and store data for further analysis. This can still be done on paper but most of the time a digital system will be used. This provides the advantage of storing data immediately into the underlying DB without the need for tedious manual operations. Collecting data in a clinical trial is done within the medical file of the patient before entering the data into the CRF; this implies the CRF can be verified and checked for inconsistencies. Every change in the system must be captured and should be traceable and accounted for by a registered user (track changes). Registered users should be listed on the delegation of authority document specifying the appointed tasks. Notwithstanding these precautions, it is important to emphasize that the PI is the one with the overall responsibility.

Collection of data on safety, such as **adverse events** (AEs) and concomitant medication (conmed) should be outlined in the protocol. Due to the nature of the ATMP/HCTP and the procedure required to apply the ATMP/HCTP, the protocol needs to be very clear on criteria used to define an AE, as opposed to what is considered an expected consequence of the procedure. Not only for the AEs but also for the conmed, when an operative technique is used to administer the ATMP/HCTP it is required to have all anesthetics (local or general) recorded or entered in the CRF.

An important point to consider when developing a CRF and DB is the privacy of the human subject. In several countries, a privacy commission has been installed by the government to follow-up on measures taken to protect the integrity of the participants and to approve the way data collection will be done. Since 2016, this is coordinated in Europe by the General Data Protection Regulation (GDPR, EU 2016/679) and controlled by the European Data Protection Board.

21.5.6 Institutional Review Board/Independent Ethics Committee and Competent Authority

When all logistics are in place, the next important step to take before entering patients in a clinical protocol is to obtain approval from an Institutional Review Board (IRB) (USA) or ethics committee (EC) (EU) as well as the national CA. The priority for both the CAs and the IRB/EC is to protect participants in a clinical trial. While it is impossible to completely split the tasks, the focus of CAs will naturally lie with regulatory compliance, adherence to guidelines, and manufacturing details, whereas the ECs will focus on ethical issues and clinical practice.

For Europe the new Clinical Trial Regulation (CTR, Regulation (EU) N°536/2014) is in place since January 31st 2022, replacing directive EC/2001/20. From January 31st 2023 all new trials have to be submitted according to the CTR using a single online application, the Clinical Trials Information System (CTIS), on which one complete package can be uploaded for CA as well as independent EC approval. So prior to the Regulation, clinical trial sponsors have to submit clinical trial applications separately to national CAc's and EC's in each country to gain regulatory and ethical approval to run a clinical trial. Now under the CTR, this process is integrated into one submission process. Via the CTIS, authorization is granted to run a clinical trial in several European countries.

For CA/EC submission, a package must be submitted by the sponsor of the trial. This package should contain the final protocol, the final informed consent form in all languages needed, evidence of

insurance/indemnity, all documents that are patient-related (such as diary cards), IB, written statements of all study centers, curriculum vitae and GCP certificates of the investigator(s), contract(s), and registration form to the CA (EudraCT in Europe). The submitted written statement in the submission package is a statement in which every investigator and every center confirms their ability to perform the protocol and determine whether the required resources are available. In case of ATMP's this is important because frequently multiple disciplines will be involved in the execution of this type of trial.

The CA/EC has 3 calendar days to check if the submission is conform to the requirements. The timeline for a single opinion is a maximum of 60 days. In case of ATMPs/HCTPs, the period to decide for an EC can be extended by 30 days, and in case extra consultations are required by a further 90 days. The independent EC must give an opinion and has one time the opportunity to request more information. In case of amendments to an approved protocol, a new package has to be submitted via CTIS for independent EC opinion.

For the US, this process is regulated by the Code of Federal Regulations (45 CFR 46) where the policy on IRBs is described.

21.5.7 Monitoring, audits, and inspections

Measures must be taken to assure quality and reliability of the registered data and to comply with GCP.

For internal quality assurance, **standard operating procedures** (SOPs) are highly recommended to describe how all procedures, ranging from obtaining informed consent to taking a blood pressure, are done on site and for the specific protocol. When SOPs are in place, everyone at site must adhere to them. Beside the description of how to handle certain procedures, SOPs also describe which actions must be taken when a procedure is done differently.

In addition, external quality assurance should be in place. The first step in this external quality assurance is a monitoring plan to check for consistency between the data entered in the CRF/DB and the source data in the subject file. This quality control is important to make sure the outcome of an RCT is reliable and can be trusted. On top of this monitoring plan, audits need to be organized by an independent organization appointed by the sponsor.

Regulatory agencies will organize inspections to check the processes of data collection and the validity of the data collected rigorously. These inspections can occur in the EU during the clinical trial stage and during an MA. In the United States, inspections are usually done at the prelicensing stage (preapproval inspection), but in some cases may be done for cause.

21.5.8 Sponsor

The sponsor of the CT can be, for instance, a private company, a governmental agency, an academic institution, or an individual. According to GCP, the sponsor organizes a clinical trial and takes ultimate responsibility for the quality and integrity of the trial data. The sponsor is not necessarily the PI, and cannot be disqualified, in contrast to the PI. Detailed sponsor responsibilities on the conduct of a trial can be found in the GCP guidelines.

For very innovative products, such as ATMPs/HCTPs, the sponsor will often be a university or an academic research unit who also takes on the role of manufacturer of these products.¹²

21.6 Special points to consider

21.6.1 Define the patient

As the therapeutic effect and potential toxicity of living cell-based products are particularly sensitive to the microenvironment they are intended for in view of donor–host interactions, defining the patient and thus the environmental effects are of great importance. Therefore, testing the products in the pre-clinical phase in models that reflect the clinical situation, and thus the proper environment, is highly desirable. In addition, the handling of the product will be guided by the surgical procedures required and thus needs to be mimicked in the preclinical studies. Furthermore, other factors that may be of importance include, but are not limited to, the age and gender of the patient and the presence of **comorbidities** such as diabetes or other diseases.

21.6.2 Manufacturing challenges and up-scaling

Importantly, the entire manufacturing process needs to be sterile from the procurement of a tissue sample to the final tissue-engineered product. Products containing living cells cannot be subjected to terminal sterilization, so contamination with pathogenic microorganisms will always incur a significant risk beyond that intrinsic to the test article itself.

Entering the human model implies a treatment scale largely exceeding the scale of small animal models. Therefore, allometric scaling needs attention, and it is imperative that the preclinical studies in larger animal models address this appropriately, as mentioned previously. The scale up also includes scaling up the manufacturing process (e.g., producing 4×10^8 cells, instead of 4×10^6 cells) and the adaptation of the surgical procedures and instrumentation. The complexity of up-scaling is frequently underestimated and leads potentially to nonrobust products of inferior quality whose survival after implantation may be negatively affected by its increased size merely because of mass transport limitations.

It is worth mentioning that, if the manufacturing process has been established using cells of healthy donors, verification is essential to ensure patient cells will behave comparably, during manufacture, shelf life, and transport. Regulatory bodies strongly encourage sponsors to perform trials intended to provide data supporting efficacy to be done with the to-be-marketed product, since analytical studies generally cannot demonstrate equivalence adequately.

21.6.3 Exploratory trials in the academic environment

Academic research is of invaluable importance in this quickly evolving field. Typically, exploratory clinical trials in an academic setting can move the field forward. However, this academic research often cannot provide efficacy and safety data collected in rigorous multicenter pivotal clinical trials. The cost for the development of treatments to live up to standards set out by the regulators and to provide a well-defined, quality product, produced within a GMP approved facility, is high and independent academic research is mostly not able to provide the resources required, with some exceptions for orphan indications. For this, the **Hospital Exemption** was introduced in Europe.

21.6.4 Hospital exemption (EU only)

When ATMPs are used in clinical trials to obtain an MA, the pathway of the central filing must be followed. Only two exemptions are foreseen in regulation 1394/2007, firstly concerning products that were legally on the market before the regulation became applicable and secondly the “hospital exemption.” ATMPs already on the market had to be compliant with the regulation no later than December 30th, 2012 or they were withdrawn. The “hospital exemption” to the centralized licensing procedure can be used to apply patient-tailored ATMPs not intended to be marketed and prepared on a nonroutine basis according to specific quality standards used within an EU member state in a hospital by a medical practitioner upon prescription. This rule enables patients to receive ATMPs when no centrally authorized medicinal product is available. Since the hospital exemption is considered a purely national framework, the interpretation and implementation of the rules differ in member states. This might be one of the reasons why the topic of the hospital exemption triggered the most responses and conflicting views in the public consultation on the 5-year review of the ATMP regulation. Intensive negotiations between the public and private sector in Europe are ongoing to harmonize the rules and create a level playing field; recent updates are published on the EMA website.

21.6.5 Voluntary harmonization procedure (EU only)

The European Voluntary Harmonization Procedure was introduced in 2009 as a pilot for the future joint assessment of clinical trials by concerned member states. It was established by the Clinical Trial Facilitation Group, a working group of the Heads of Medicines Agencies and consists of a joint assessment phase for multinational clinical trials by the competent authority of concerned member states followed by an accelerated national authorization procedure. It is open to all trials involving two or more member states willing to participate and involves a validation phase and a joint assessment phase (voluntary) followed by the legally required national submission. The benefit for the applicant lies in the provision of joint comments, which facilitates dossier harmonization across member states.

21.6.6 Combination products

Combination products typically may contain two or more of the following: cells, scaffolds or biomaterials, and/or soluble growth factors. This implies not only a strict characterization of the starting materials such as the cells, the biomaterials, and growth factors, but also in-depth studies of the combination. For instance, cell behavior may be strongly affected when deposited on a biomaterial, and potency assays for cells delivered on a scaffold may thus be different when the same cells are injected as cell suspensions. Also, distinct lots of biomaterials may be quite different when looking at the material characteristics such as pore size, and that may affect the biological behavior of the combination product. Importantly, the manufacturing process to make the combination product is quite challenging and needs special attention and validation, with the need to demonstrate that data obtained in later stages of the development are comparable to those obtained in the early phases. In any case, it is strongly recommended to establish the manufacturing process that will be used for the commercial product prior to initiation of the pivotal clinical studies intended to generate efficacy data to support licensure.

21.7 Future perspective

As described, the process of developing a tissue-engineered product, in particular an ATMP/HCTP, is challenging, especially because the field is evolving so rapidly. New regulation came into effect in Europe for medical devices in May 2021, raising the bar for clinical investigations. In recent years, much effort has been directed at creating a framework for development of ATMPs/HCTPs, but during this process new hurdles have arisen. From the regulatory point of view, FDA, as well as EMA, mechanisms have been created to facilitate the development of TE ATMPs/HCTPs. The recent creation of an RMAT designation in the USA/FDA is another example. Nevertheless, notwithstanding the unique nature of each ATMP/HCTP, the regulation allows great flexibility to conduct proper investigations in this field. The most important message that can be given is to reach out to the agencies very early in the development for scientific and regulatory advice.

We further believe it is critical to respect the basic rules and regulations for drug development, which has been informed extensively—and in salient cases painfully—by the historical incidents and resulting experience with the regulation and clinical development that evolved over the last century. This includes GCP, the “first do not harm” principle, Good Manufacturing Practices, and proper assessment of efficacy. Indeed, providing a treatment that does not work also always incurs a safety issue, since for many of these products, it is impossible to eliminate the possibility of introducing infectious agents and consequent deleterious effects for the subjects.

Finally, special attention in this field needs to go to informed consent. Indeed, as for many of these approaches, there is a poor understanding of the underlying mechanisms of action, and such aspects as drug (cells) disposition are not yet fully appreciated. Therefore, the subject needs to be a “full” partner in these novel treatment approaches. In contrast to a device, once the cells are in, we cannot remove them, and some of the cell products may persist for the lifespan of the patient, thereby allowing the possibility for unforeseeable late safety events. Because of dedicated research and development, some treatments have ultimately reached the clinic. This has brought important progress for patients in the past, and will continue to do so, even with a limited understanding of the new treatment approach. The development of new enabling technologies and a stimulating and collaborative regulatory environment will be key factors in the success of these approaches.

Summary

- Clinical translation involves a preclinical as well as a clinical dossier.
- Preclinical studies need to be evaluated for their appropriateness for a particular clinical indication and may include small and/or large animal models relevant to the clinical situation to be addressed.
- Large animal models may provide essential information on feasibility and safety aspects, particularly when anatomic similarity and/or allometric scaling considerations are critical.
- Exploratory trials in the human model may identify unexpected behavior of tissue-engineered products.
- Exploratory trials may provide information on potential efficacy and proof of principle.
- A regulatory framework for TE products has evolved around the world.
- Regulatory bodies should be involved as early as possible, not only for product designation but also for guidance in nonclinical and clinical development.

Continued

Summary—continued

- Pitfalls for the clinical development of TE products include dosing, defining the comparator, randomization, blinding, endpoints, and standardization of patient care and follow-up.
- Upscaling not only implies allometric up scaling but also scale up of manufacturing and surgical approaches.
- A patient-centered approach and patient consent are essential.

Classical experiment

A Cell-based combination product for the repair of large bone defects.

This preclinical study on healing of large nonhealing long bone defects by use of a cell-based combination product is a series of large animal experiments to demonstrate the capacity of restoring large bone defects in conditions that simulate the real clinical situation.

Despite the fact that a lot of effort is put in the development of cell-based ATMPs, the scaling to large bone defects remains a problem. Lammens et al. showed that cell-based implants had good biological potential in an ectopic nude mouse model and scaled this to a more clinically relevant animal model, i.e., critical size large tibial bone defects and nonunions in sheep.

For this trial 72 sheep were divided in a fresh defect or a biologically exhausted defect group, the latter model representing the nonunion patient. Both groups were treated with scaffold only, scaffold coated with BMPs, or cell-based ATMPs with BMPs.

Periosteal cells were harvested during the surgical procedure to create the defect and used for cell culture. After expansion, the cells were harvested and implants/ATMPs

were assembled and fitted into the defects. Bone healing/formation and safety were evaluated.

In fresh bone defects, scaffold only revealed success in half of the cases and enrichment with bone morphogenetic proteins (BMPs) improved the success rate, while cell-based ATMP did not add additional value. In biologically exhausted defects, a nonunion model which reflects closer the clinical situation, scaffolds, with or without BMPs, did not result in defect bridging. In contrast, a cell-based combination product in this group resulted in successful healing in half of the cases, showing the potential of the latter.

In conclusion, preclinical studies are challenging and not necessarily predictive of the outcomes in the patients, as shown in this article comparing fresh defects with biological exhausted defects.

It needs to be emphasized that the scientist's interest in bone formation does not always match the clinician's need for a functionally repaired bone. Discussion with clinical opinion leaders, ethics committees, and agencies is important to translate such results to the clinical situation.

Lammens J, et al. *Bone*. 2020;138:115511.

State-of-the-art experiment

Autologous Chondrocyte Implantation as an example for clinical translation in Advanced Therapy

Medicinal products

ACI and more specifically the Characterized Chondrocyte Implantation is an example for clinical translation and implementation of a new ATMP/HCTP in the field of TE. This example shows the different steps to take and pitfalls to avoid to obtain a marketing authorization (MkA) for this type of product.

Despite the fact that the technique of ACI has been in use since 1994, Knutsen et al.¹³ were the first to perform an RCT to test the technique versus MF, a treatment approach regarded as standard of care by most surgeons. This well-designed RCT aimed to compare the outcome of the treatment approaches by examining the ability of the product to promote hyaline cartilage formation/repair and symptomatic relief. Within this trial, attention was paid to the design elements of a classical clinical trial: prospective with randomization, defined scoring of relevant outcome parameters, training of the surgeons, and harmonization of the rehabilitation program.

Saris et al. [2008] took this a step further and introduced a more detailed characterization of the cell product. Indeed, in vitro expansion of articular chondrocytes leads to dedifferentiation, the cells losing some their very specific functions. In this trial, special attention was given to the manufacturing process, and to define and characterize the final cell product to make sure it consisted of a specific dose of viable chondrocytes able to produce hyaline

cartilage. There was advanced preclinical training of the surgeons and a well-defined rehabilitation and follow-up protocol. There were fixed audits to verify compliance with the protocol.

Despite the attention paid to the design of the RCT, pitfalls arose during the execution of the trial. The most striking example was the choice of MF as the standard of care/comparator: the existence/acceptance of MF as standard of care in daily practice did not provide evidence that it was proven to be superior to placebo or to a nonsurgical, conservative approach. In the course of subsequent literature searches, discussions with the orthopedic community, or with regulatory bodies, it turned out that no RCTs had demonstrated superiority of MF convincingly. Indeed, the natural course of symptomatic joint surface defects in the knee appeared to be insufficiently documented. Therefore, the effectiveness of MF had to be evaluated in addition to the proposed experimental therapy.

In conclusion, clinical translation of ATMPs/HCTPs is challenging but, as for all new conventional drugs, necessary to obtain Marketing Approval. When new treatment approaches are investigated, an in-depth analysis is needed not only with respect to commonly accepted clinical practice, but also to critically examine the evidence supporting current clinical practice.”

Saris DBF, et al. Characterized chondrocyte implantation results in better clinical outcome at 36 months in a randomized trial compared to microfracture. *Am J Sports Med.* 2009;37(suppl 1): 10S–19S.

21.8 Recommended literature

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2. Vanlauwe J, Saris DB, Victor J, Almqvist F, Bellemans J, Luyten FP, TIG/ACT/01/2000& EXT Study Group. Five-year outcome of characterized chondrocyte implantation versus microfracture for symptomatic cartilage defects of the knee: early treatment matters. *Am J Sports Med.* 2011;39(12): 2566–2574.

21.8.1 Recommended websites

1. <http://www.eunetha.eu/>
2. <http://www.ema.europa.eu/>
3. <https://www.ema.europa.eu/en/human-regulatory/overview/advanced-therapy-medicinal-products-overview>
4. <https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products>
5. <http://www.fda.gov/>
6. <http://www.ema.europa.eu/ema/>
7. <https://www.astm.org/COMMIT/SUBCOMMIT/F0444.htm>
8. <http://www.ich.org/>
9. <http://www.eunetha.eu/>
10. http://ec.europa.eu/health/human-use/clinical-trials/index_en.htm

21.9 Assessment of your knowledge

- (a) Answer the following questions to assess your command on terminology, facts, concepts, and theories learned in this chapter:
 1. Explain the Medical Devices and classification in Classes I-IIa-IIb-III.
 2. Explain the IMDD-investigational medical device dossier in terms of its content.
 3. In what year did the new Medical Devices Regulation (MDR) go into effect?
 4. What is the biggest change/benefit in the new MDR compared to the previous regime?
 5. To whom/which organization does the manufacturer of medical devices report to?
 6. Which products are understood as ATMPs and why?
 7. When was the new ATMP regulation introduced? By what organization?
 8. Difference between sponsor and principal investigator of a clinical study; who is liable in case of major Serious Adverse Events, including fatalities?
 9. Explain the distinction between efficacy and effectiveness of a new therapy
 10. What documents need to be prepared/signed of before a clinical trial can be initiated?
 11. Since double blinding a trial intervention is not always feasible, which stakeholders should be consulted at the study design phase?
 12. Explain the difference between a directive and a regulation.
- (b) Answer the following questions to assess your ability to apply the concepts and theories learned in this chapter in real life, clinical, and scientific situations.
 1. Discuss choice of appropriate **preclinical animal models** for joint surface repair
 2. Discuss choice of **preclinical animal models** for the treatment of large bone defects
 3. Discuss/define the **timelines for time to market** of a novel bone defect healing strategy with a medical device versus an ATMP with as example:
 - a. a bioceramic only
 - b. a bioceramic coated with a growth factor
 - c. a bioceramic with growth factor and expanded bone marrow stromal cells seeded on it.

4. Clinical trial design:
 - a. Why is it important to compare novel treatment strategies to the standard-of-care?
 - b. What if the standard-of-care has never been investigated properly versus placebo control?
 - c. What consideration should be given to defining the control?
5. What do we mean by **unmet medical need**? How do you assess the unmet medical need?
6. Discuss the importance of **manufacturing** and **scaling** of a novel device or ATMP
7. What are the required characterizations of the product/cells (ATMPs/HCTPs) intended for the treatment that should be evaluated to ensure that the final product can be manufactured robustly and reproducibly?
8. Can you comment on challenges encountered in study design/or conduct for ATMPs regarding choice of outcome measures and subject care and follow-up?
9. What kind of monitoring and audits are warranted, and why?
10. What items should be included in the informed consent, what is the importance of the informed consent process, and how would you document this process?
11. Please discuss short and long-term efficacy based on examples

Challenge-based learning

Building a preclinical research plan to enter into a clinical trial to heal peripheral nerve damage

Note for teachers: A challenge-based learning (CBL) user guide can be found at www.jandeboerlab.com/TissueEngineering with instructions and tips to run an effective CBL teaching session.

Background and vision

The ultimate goal of TE is to apply tissue-engineered products in the clinic to cure patients. Product development has its classical trajectory going from in vitro experiments to preclinical proof of principle in animals and finally a carefully designed clinical study in man. To move tissue engineered products to the market, one has to prove that these products are feasible, effective, and safe. The decision of the regulatory bodies to approve a clinical trial to test a tissue-engineered product depends on research data that scientists have acquired over the years. It is our vision that every scientist should have a well-designed preclinical research plan to accelerate and expedite the step into clinical research.

Motivation and stakeholders

Exploratory clinical trials provide information on efficacy of tissue-engineered product. A clear and detailed regulatory framework for TE products needs to be followed to enter into clinical experimentation. This regulatory framework has evolved over time and now many countries have

comparable regulatory requirements to allow the use of TE products around the world. Therefore, tissue engineers should consult the regulatory bodies as early as possible to develop tissue-engineered products. Their definitions, regulations, and documentation provide guidance in preclinical and clinical development. To build a preclinical research plan, researchers should consider the needs, requirements, and regulatory, financial, and technical boundary conditions defined by stakeholders such as all scientists involved in product development, the patients who are treated with the product, and clinicians who apply it and the product manufacturers.

Problem definition

The European Medical Agency (EMA) has issued regulatory guidelines for the implementation of tissue-engineered products, but the scientific path toward the approval of the first clinical trial depends on the type of product one wants to put in the market. There is a lack of awareness in the TE community to carefully integrate EMA regulation as the basis of the design of preclinical research.

Challenge

To design the preclinical research plan to convince EMA to test a therapeutic strategy to heal a critical-sized peripheral nerve defect with a biomaterial of choice and iPSC-derived neurons.

Continued

Challenge-based learning—continued

Learning framework

Reading the Clinical Translation chapter and related literature will help you to understand:

1. How EMA decides on the approval of a clinical trial.
2. The set of regulations EMA uses to make the decision.
3. The current bottlenecks and most common challenges in the approval of tissue engineered products.

For a more focused examination of the challenge, read scientific literature and create a mind map to include information about the following:

4. The phases of research prior to a clinical trial.

5. The critical and quantifiable experimental evidence needed to make the transition from in vitro to preclinical research.
6. The safety and efficacy data required by EMA to enter into a clinical trial.

End product

A 3-min video explaining the solution of your challenge. Please include your motivation and the steps to execute your solution.

© Jan de Boer. CBL available for classroom use and CBL videos and can be found at www.jandeboerlab.com/TissueEngineering.

21.10 Glossary

Active substance file holds confidential intellectual property or ‘know-how’ of the manufacturer of the active substance of a drug.

Advanced Therapeutic Medicinal Products (ATMPs) are advanced therapeutic drugs that are based on cell therapy or gene therapy (sometimes in combination with a medical device).

Adverse events are undesired medical occurrences in a patient or clinical investigation subject after the administration of a pharmaceutical product. An adverse event or effect does not necessarily have a causal relationship with the treatment.

Allometric scaling is a prediction method to provide a “sneak peek” at how a product might behave in humans before any clinical studies are conducted.

Biodistribution is where compounds of interest travel in an experimental animal or human subject.

Biomechanical limitations pertain to the geometric properties of the musculoskeletal system and the stiffness properties of the tissues within this system that affect the movement of an individual.

Cellular and tissue-based products are products containing or consisting of (human) cells or tissues which are intended for implantation, transplantation, infusion, or transfer to a human recipient.

Clinical directive is a European Union directive aimed at facilitating the internal market of medicinal products within the European Union.

Comorbidities are the presence of one or more additional conditions often cooccurring with a primary condition

Comparator is the currently accepted standard treatment for the specific clinical problem to be investigated.

Double-blind process is a step in experimental design in which neither the participants nor the experimenters know who is receiving a particular treatment.

Endpoint (in clinical research) is a disease state, symptom, or sign that constitutes one of the target outcomes of the trial or its participants.

Good Clinical Practice (GCP) is an international quality standard, which governments can use to transpose into regulations for clinical trials involving human subjects.

Health Technology Assessment (HTA) is a multidisciplinary process that uses systematic and explicit methods to evaluate the properties and effects of a medical technology.

Hospital Exemption is a pathway (only in Europe) to enable patients to receive an Advanced Therapy Medicinal Product (ATMP) from a clinician under controlled conditions in cases where no centrally authorized medicinal products are available for an indication with a high unmet medical need.

Informed consent is a principle in medical ethics and medical law that consists of sharing sufficient information with a patient so he/she can make a free and well-informed decision about his/her medical care.

Market Approval is the process of reviewing and assessing the evidence to support the use of a medicinal product, such as a drug, in relation to its marketing. This process ends by granting a license to sell the product.

Medical device is any instrument, apparatus, appliance, software, or material used alone or in combination with another device for diagnostic and/or therapeutic uses.

Mode of action describes how a treatment works, what is the underlying mechanism resulting from the exposure of a living organism to a substance.

Notified Bodies are organizations in the European Union designated by a member state to assess the conformity of certain products, before being placed on the EU market, with the applicable essential requirements including a technical dossier and data management systems.

Phase I, human pharmacology (phase) is the first stage of testing in human subjects, designed to test the safety, side effects, dose, and formulation method for the drug.

Phase II, therapeutic exploratory (phase) is performed on larger group of human subjects (50–300) to assess how well the drug works and the optimal dose and to continue Phase I safety assessments in a larger group of volunteers and patients.

Phase III, therapeutic confirmatory (phase) also called pivotal trial as definitive assessment of drug efficacy and safety in comparison with a current ‘gold standard’ treatment.

Phase IV studies are done to assure long-term safety and effectiveness of the drug, vaccine, device, or diagnostic test.

Potency assay is the quantitative measure of the presumed biological activity relevant to the outcome; ideally it measures the ability of the product to elicit a specific clinical response.

Preclinical is a stage of research that begins before clinical trials (testing in humans) and during which important feasibility, iterative testing, and drug safety data are collected, typically in in vitro/ex vivo models and in vivo laboratory animals.

Protocol is the key document that contains all information on the conduct of the specific trial.

Quality Attributes are measurable or testable properties of a process used to indicate how well the process performs to manufacture a well-defined product.

Quality management system is a collection of processes focused on consistently meeting customer and product requirements and enhancing their satisfaction.

Randomization is a sequence of random variables describing a process whose outcomes do not follow an evolution described by probability distributions.

Randomized Controlled Trials are a form of clinical trial used to control factors not under direct experimental control.

Example Clinical trials that compare the effects of drugs, surgical techniques, medical devices, diagnostic procedures, or other medical treatments.

Regenerative medicine deals with the process of replacing, engineering, or regenerating human or animal cells, tissues, or organs to restore or establish normal function.

Regulation in the context of marketing a product harmonizes the assessment and supervision of processes for clinical trials throughout the European Union, via a Clinical Trials Information System.

Staged clinical trials are distinct phases of clinical research in which scientists conduct experiments with a health intervention to obtain sufficient evidence for a process considered effective as a medical treatment.

Standard operating procedures (SOPs) are sets of step-by-step instructions compiled by an organization to help workers carry out routine operations to become user independent, thus robust, and reproducible.

Statistical power usually expressed in percentages, is the probability that the test correctly rejects the null hypothesis when a specific alternative hypothesis is true.

Technical product documentation refers to any document that explains the use, functionality, production, or architecture of a product.

Technology Readiness Level(s) (TLR) is a method for estimating the technical maturity of a technology during its development.

Tissue engineering is a discipline that uses a combination of cells, engineering, materials methods, and suitable biochemical and physicochemical factors to restore, maintain, improve, or replace different types of biological tissues.

© Jan de Boer. All glossaries can be found at www.jandeboerlab.com/TissueEngineering.

Some of these definitions were freely obtained and paraphrased from Wikipedia and Google.

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TISSUE ENGINEERING

THIRD EDITION

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Tissue engineering (TE) is a rapidly growing field because technological advancements in the field of 3D tissue engineering meets the enormous demand for regeneration due to ageing and trauma. *Tissue Engineering, Third Edition* is completely revised and goes from basic disciplines (stem cells, biomaterials) towards tissue engineering of specific tissues and towards clinical application. Key chapters are updated with the latest discoveries. Written in a scientific language that is easily understood by undergraduate and graduate students in basic biological sciences, bioengineering, and basic medical sciences, as well as researchers interested in learning about this fast-growing field.

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ACADEMIC PRESS

An imprint of Elsevier

elsevier.com/books-and-journals

ISBN 978-0-12-824459-3



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